(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
9 January 2003 (09.01.2003)

PCT

(10) International Publication Number
WO 03/002595 A2

(51) International Patent Classification[7]: C07K 7/08, A61K 38/04, 38/08, 35/00, A61P 35/04

(21) International Application Number: PCT/EP02/07129

(22) International Filing Date: 27 June 2002 (27.06.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

01114796.4	27 June 2001 (27.06.2001)	EP
101 50 203.6	12 October 2001 (12.10.2001)	DE
101 54 689.0	9 November 2001 (09.11.2001)	DE
60/360,909	28 February 2002 (28.02.2002)	US

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 03/002595 A2

(54) Title: NEW DIPEPTIDYL PEPTIDASE IV INHIBITORS AND THEIR USES AS ANTI-CANCER AGENTS

(57) Abstract: The present invention provides new uses of DPIV-inhibitors of the present invention, and their corresponding pharmaceutically acceptable acid addition salt forms, for treating conditions mediated by DPIV or DPIV-like enzymes, such as cancer and tumors. In a more preferred embodiment, the compounds of the present invention are useful for the treatment of metastasis and tumor colonization.

New Dipeptidyl Peptidase IV Inhibitors and Their Uses As Anti-Cancer Agents

Field Of The Invention

The present invention relates to inhibitors of dipeptidyl peptidase IV and dipeptidyl peptidase IV-like enzyme activity and, more particularly, pharmaceutical compositions containing said compounds, and the use of said compounds for the treatment of cancer and tumors. The present invention especially provides a method for the inhibition of metastasis and tumor colonization.

Background Art

Dipeptidyl peptidase IV (DPIV) is a serine protease which cleaves N-terminal dipeptides from a peptide chain containing, preferably, a proline residue in the penultimate position. Although the biological role of DPIV in mammalian systems has not been completely established, it is believed to play an important role in neuropeptide metabolism, T-cell activationand the entry of HIV into lymphoid cells.

Likewise, it has been discovered that DPIV is responsible for inactivating glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide also known as gastric-inhibitory peptide (GIP). Since GLP-1 is a major stimulator of pancreatic insulin secretion and has direct beneficial effects on glucose disposal, in WO 97/40832 and US 6,303,661 inhibition of DPIV and DPIV-like enzyme activity was shown to represent an attractive approach for treating non-insulin-dependent diabetes mellitus (NIDDM).

The present invention provides a new use of DPIV-inhibitors for the prophylaxis and treatment of conditions mediated by inhibition of DPIV and DPIV-like enzymes, in particular the prophylaxis and treatment of cancer and tumors and the prophylaxis and inhibition of metastasis and tumor colonization, and pharmaceutical compositions e.g.

useful in inhibiting DPIV and DPIV-like enzymes and a method of inhibiting said enzyme activity.

This invention relates to a method of treatment, in particular to a method for the prophylaxis and treatment of cancer, tumors, metastasis and tumor colonization and to compounds and compositions for use in such method. Dipeptidyl peptidase IV (DPIV) is a post-proline (to a lesser extent post-alanine, post-serine or post-glycine) cleaving serine protease found in various tissues of the body including kidney, liver, and intestine.

It is known that DPIV-inhibitors may be useful for the treatment of impaired glucose tolerance and diabetes mellitus (International Patent Application, Publication Number WO 99/61431, Pederson RA et al, Diabetes. 1998 Aug; 47(8):1253-8 and Pauly RP et al, Metabolism 1999 Mar; 48(3):385-9). In particular WO 99/61431 discloses DPIV-inhibitors comprising an amino acid residue and a thiazolidine or pyrrolidine group, and salts thereof, especially L-*threo*-isoleucyl thiazolidine, L-*allo*-isoleucyl thiazolidine, L-*threo*-isoleucyl pyrrolidine, L-*allo*-isoleucyl thiazolidine, L-*allo*-isoleucyl pyrrolidine, and salts thereof.

Further examples of low molecular weight dipeptidyl peptidase IV inhibitors are agents such as tetrahydroisoquinolin-3-carboxamide derivatives, N-substituted 2-cyanopyroles and –pyrrolidines, N-(N'-substituted glycyl)–2-cyanopyrrolidines, N-(substituted glycyl)-thiazolidines, N-(substituted glycyl)-4-cyanothiazolidines, amino-acyl-borono-prolyl-inhibitors, cyclopropyl-fused pyrrolidines and heterocyclic compounds. Inhibitors of dipeptidyl peptidase IV are described in US 6,380,398, US 6,011,155; US 6,107,317; US 6,110,949; US 6,124,305; US 6,172,081; WO 95/15309, WO 99/61431, WO 99/67278, WO 99/67279, DE 198 34 591, WO 97/40832, DE 196 16 486 C 2, WO 98/19998, WO 00/07617, WO 99/38501, WO 99/46272, WO 99/38501, WO 01/68603, WO 01/40180, WO 01/81337, WO 01/81304, WO 01/55105, WO 02/02560 and WO 02/14271, the teachings of which are herein incorporated by

reference in their entirety, especially concerning these inhibitors, their definition, uses and their production.

The term DPIV-like enzymes relates to structurally and/or functionally DPIV/CD26-related enzyme proteins (Sedo & Malik, Dipeptidyl peptidase IV-like molecules: homologous proteins or homologous activities? Biochimica et Biophysica Acta 2001, 36506: 1-10). In essence, this small group of enzymes has evolved during evolution to release H-Xaa-Pro-Dipeptides and H-Xaa-Ala-Dipeptides from N-terminus of oligo- or polypeptides. They show the common feature that they accomotate in the Pro-position also Al, Ser, Thr and other amino acids with small hydrophobic side-chains as, Gly or Val. The hydrolytic efficacy is ranked Pro>Ala» Ser, Thr » Gly, Val. Same proteins have been only available in such small quantities, that only the post-Pro or post-Ala cleavage could be established. While the proteins: DPIV, DP II, FAPα (Seprase), DP 6, DP 8 and DP 9 are structurally related and show a high sequence homology, attractin is an extraordinary functional DPIV-like enzyme, characterized by a similar activity and inhibitory pattern.

Further DPIV-like enzymes are disclosed in WO 01/19866, WO 02/04610, WO 02/34900 and WO02/31134. WO 01/19866 discloses novel human dipeptidyl aminopeptidase (DPP8) with structural und functional similarities to DPIV and fibroblast activation protein (FAP). WO 02/34900 discloses a novel dipeptidyl peptidase 9 (DPP9) with significant homology with the amino acid sequences of DPIV and DPP8. WO 02/31134 discloses three DPIV-like enzymes, DPRP1, DPRP2 and DPRP3. Sequence analysis revealed, that DPRP1 is identical to DPP8, as disclosed in WO 01/19866, that DPRP2 is identical to DPP9 and that DPRP3 is identical to KIAA1492 as disclosed in WO 02/04610.

<u>DPIV and DPIV-like enzymes in immunophysiology and cancer</u>

Dipeptidyl peptidase IV (DPIV; EC 3.4.14.5; CD26) CD26 is a M_r 110,000 surface glycoprotein with an array of diverse functional properties that is expressed on

a number of tissues, including epithelial cells and leukocyte subsets (Mentlein, 1999). Furthermore, it is a membrane-associated ectopeptidase which exhibits its activity in its extracellular domain and is able to cleave N-terminal dipeptides from polypeptides with either L-proline or L-alanine in the penultimate position.

Cancer pathomechanisms

Cancer is a group of over 150 diseases characterized by the uncontrolled growth of abnormal cells in the body. Normal cells can become abnormal when they are exposed to carcinogens such as radiation or particular drugs or chemicals. They can also turn malignant (cancerous) when they are attacked by certain viruses or when some not-yet-fully-understood internal signal occurs. Once cells become malignant, they multiply more rapidly than usual. Then they often form masses called tumors that invade nearby tissue and interfere with normal bodily functions. Cancer cells also have a tendency to spread to other parts of the body, where they may form a secondary tumor.

Mechanisms of metastasis

The outcome of cancer metastasis depends on multiple interactions within the target tissue and depends on the microenvironment including cellular adhesion molecules, chemokines, or hydrodynamic effects and many other factors. In addition, a very rapid attraction of leukocytes and specific cellular responses at the tumor sites may play a critical role in the early host defense against cancer. These early changes may be of critical importance for the outcome of metastatic disease and may extend the present understanding of the host resistance against metastasis.

WO 99/47152 discloses a method of suppressing the malignant phenotype or inducing apoptosis of cancer cells in a subject, comprising introducing into the cancer cell an amount of a nucleic acid encoding a dipeptidyl peptidase IV protein or fibroblast activating protein-α, thereby suppressing the malignant phenotype of the cancer. WO 99/47152 also discloses a method of inducing expression of dipeptidyl peptidase IV or fibroblast activating protein-α in cancer cells of a subject, comprising administering to the subject a pharmaceutical composition comprising a therapeutically effective amount of an agent capable of activating transcription of the dipeptidyl peptidase IV gene or fibroblast activating protein-α gene and a pharmaceutical acceptable carrier or diluent.

Current treatments of cancer and tumor cell adhesion

Current cancer treatment regimens comprise surgery, chemotherapy, radiation therapy, and other treatment methods including immunotherapy. Immunotherapy is composed of the usage or the modification of intrinsic bodily mechanisms – in most cases immune mechanisms - to fight cancer. Chemotherapy kills cancer cells through the use of drugs or hormones. Taken either orally or through injection, chemotherapeutic agents are used to treat a wide variety of cancer. They may be given alone or in combination with surgery or radiation or both. Chemotherapy is an established way to destroy hard-to-detect cancer cells that have spread and are circulating in the body. Anemia (low number of red blood cells) is a frequent side effect of chemotherapy and may cause symptoms such as extreme tiredness, dizziness, or shortness of breath. Epoetin alfa (Procrit®, Epogen®) - recombinant erythropoletin that stimulates red blood cell production - is a prescription drug available for the treatment of chemotherapy-related anemia.

Immunotherapy uses the body's own immune system or other parts of the organism to destroy cancer cells. This form of treatment is still being intensively studied in clinical trials; it is not yet widely available to most cancer patients. The various immunological agents used include substances produced by the body (such as the interferons, the interleukins and tumor necrosis factor) and laboratory-produced

substances (such as monoclonal antibodies and vaccines). Immunological agents work in different ways and can be used independently or in combination with other forms of treatment.

Angiogenesis inhibitors as anti-metastatic drugs in immunotherapy

Angiogenesis inhibitors are drugs that block the development of new blood vessels. Solid tumors cannot grow without inducing the formation of new blood vessels. Blocking the development of new blood vessels cuts off the tumor's supply of oxygen and nutrients.

Several angiogenesis inhibitors are currently being tested in human trials. In cancerous tissue, tumors cannot grow or spread (metastasize) without the development of new blood vessels. Blood vessels supply tissues with oxygen and nutrients necessary for survival and growth.

Summary Of The Invention

The present invention provides new uses of DPIV-inhibitors of formulas 1 to 12, and their corresponding pharmaceutically acceptable acid addition salt forms for preventing and treating cancer and tumors. In a more preferred embodiment, the compounds of the present invention are useful for the prevention and inhibition of metastasis and tumor colonization.

Reduced expression of the ectopeptidase DPIV and lack of DPIV-like activity in lungs of mutant F344 rats lacking DPIV enzymic activity and expression results in reduced adhesion of cancer cells and in reduced lung metastasis. *In vivo* cell adhesion and growth of the F344 rat syngeneic mammary adenocarcinoma MADB106 was studied in F344 rats after acutal and chronic treatment with DPIV-ligands *in vivo*. Mutant F344 substrains lacking DPIV enzymic activity and wild-type-like F344 were tested. Chronic intragastric infusion of isoleucyl cyano pyrrolidine TFA and isoleucyl thiazolidine fumarate via osmotic minipumps over two weeks dose-dependently reduced the

cancer-induced weight loss and the number of tumor colonies on the lung surface. Thus, metastasis of MADB106 is reduced by chronic treatment using different DPIV inhibitors (isoleucyl thiazolidine fumarate; isoleucyl cyano pyrrolidine TFA) suggesting protective-like class effects by the two different DPIV-inhibitiors/ligands. Possibly, isoleucyl thiazolidine fumarate and isoleucyl cyano pyrrolidine TFA protect from metastasis either via interaction with cell adhesion processes, via a modification of the cellular host defense mechanisms, via modulation of angiogenesis, via direct effects on cancer cells, or via increased levels of DPIV substrates, which indirectly mediate protective-like effects.

Brief Description Of The Drawings

Fig. 1: Effect of single injection of isoleucyl thiazolidine fumarate on lung metastasis in F344 rats. Vital dye (Carboxyfluorescein; CFSE) labeled MADB106 tumor cells were injected via the lateral tail vein and lungs were collected 30min after inoculation. CFSE positive tumor cells in lungs were quantified by means of immunohistology and image analysis. Data represent means ± SEM; no significant differences vs. saline treated controls were found.

Fig. 2: Effect of single injection of isoleucyl cyanopyrrolidine TFA on tumor cell adhesion 30min after injection in F344USA rats. CFSE labeled MADB106 cancer cells were injected via the lateral tail vein and lungs were collected 30min after inoculation. CFSE positive tumor cells in lungs were quantified by means of immunohistology and image analysis. Data represent means ± SEM; significant differences vs. saline treated controls were not found.

Fig. 3: Effect of single injection of valyl pyrrolidine fumarate on tumor cell adhesion 30min after injection in F344USA rats. CFSE labeled of MADB106 adenocarcinoma cells were injected via the lateral tail vein and lungs were collected 30min after inoculation. CFSE positive tumor cells in lungs were quantified by means

of immunohistology and image analysis. Data represent means ± SEM; significant differences vs. saline treated controls were not found.

Fig. 4: Effect of chronic intragastric infusion of isoleucyl thiazolidine fumarate on body weight change in grams in F344 rats with lung metastasis. A dose dependent reduction of the loss of body weight after chronic infusion of different dosages of isoleucyl thiazolidine fumarate in F344 rats 2 weeks after injection of MADB106 tumor cells is illustrated. One factor ANOVA revealed a significant effect on body weight, which became significant in the post-hoc analysis at the 0.4mg and 4mg dosages. Data represent means ± SEM; $^{*}p<0.05$ reflecting significant differences vs. saline treated SHAM controls determined by Fisher PLSD.

Fig. 5: Effect of chronic intragastric infusion of isoleucyl thiazolidine fumarate on the number of lung tumor colonies in F344 rats. A dose dependent reduction of lung colony numbers after chronic infusion of different dosages of isoleucyl thiazolidine fumarate in F344 rats 2 weeks after injection of MADB106 tumor cells is illustrated. One factor ANOVA revealed a significant effect, which became significant in the post-hoc analysis at the 4mg dosage. Data represent means ± SEM; $^{*}p<0.05$ reflecting significant differences vs. saline treated SHAM controls determined by Fisher PLSD.

Fig. 6: Effect of chronic intragastric infusion of isoleucyl thiazolidine fumarate; isoleucyl cyanopyrrolidine TFA, and valyl pyrrolidine fumarate on the number of lung tumor colonies in F344 rats. A significant reduction of lung colony numbers after chronic infusion of isoleucyl thiazolidine fumarate and isoleucyl cyanopyrrolidine TFA in F344 rats 2 weeks after injection of MADB106 tumor cells is illustrated. Data represent means ± SEM; $^{*}p<0.05$ reflecting significant differences vs. saline treated SHAM controls determined by ANOVA and Fisher PLSD.

Detailed Description Of The Invention

The present invention relates to the area of dipeptidyl peptidase IV (DPIV) inhibition and, more particularly, to a new use of inhibitors of DPIV and DPIV-like enzyme activity for the prevention and treatment of cancer and tumors, in particular for the prevention and inhibition of metastasis and tumor colonization, and pharmaceutical compositions containing said compounds.

In contrast to other proposed methods in the art, the present invention especially provides an orally available therapy with low molecular weight inhibitors of dipeptidyl peptidase IV. The instant invention represents a novel approach for the prevention and treatment of cancer and metastatic disease. It is user friendly, commercially useful and suitable for use in a therapeutic regime, especially concerning human diseases.

Spontaneous mutations of the DPIV gene observed in substrains of F344 rats provide a model for studying the role of DPIV in tumor adhesion and colonization. The mutations in F344 rats result in a lack of DPIV enzymatic activity and are found in substrains from Germany (GER) and Japan (JAP) (Thompson et al, 1991; Tsuji et al, 1992), while rats from USA breeders show significant enzyme activity. In F344JAP rats, a G633R substitution in the DPIV protein causes markedly reduced expression of a mutant inactive enzyme (Cheng et al, 1999; Tsuji et al, 1992;), while the other DPIV negative F344GER substrain expresses a non-active mutant enzyme (Thompson et al, 1991).

On the basis of these findings, the investigation of the role of DPIV expression and enzymic activity in cancer according to the present invention revealed that the oral administration of DPIV inhibitors results in a decrease of lung metastasis and colonization.

The goal of the present invention is the development of dipeptidyl peptidase IV inhibitors and/or ligands, which display a high bioavailability. In another preferred embodiment, the present invention provides DPIV inhibitors, which have an exactly predictable activity time in the target tissue.

Examples for target specific, orally available low molecular weight agents are prodrugs of stable and unstable dipeptidyl peptidase IV inhibitors of the general formula A-B-C, wherein A represents an amino acid, B represents the chemical bond between A and C or an amino acid, and C represents an unstable or a stable inhibitor of dipeptidyl peptidase IV respectively. They are described in WO 99/67278 and WO 99/67279 the teachings of which concerning the provision, definition, use and production of the prodrugs are herein incorporated by reference in their entirety. Especially the detailed definitions of A, B and C are herein incorporated by reference.

The present invention relates to a novel method, in which the reduction of activity in the enzyme dipeptidyl peptidase (DPIV or CD 26), or of DPIV-like enzyme activity, or where binding of a DPIV specific ligand exerts tumor suppressive or immunostimulating effects in the organisms of mammals induced by effectors of the enzyme and leads as a causal consequence to a reduced growth or adhesion of cancer cells. Such treatment will result in a reduction or delay of cancer cell adhesion (metastasis) or the growth of tumor. As a consequence mammals bearing cancer will benefit from the treatment with inhibitors of DPIV a DPIV-like enzyme activity.

The method and use according to the present invention for preventing and treating cancer and related disorders in an animal, including humans, in need thereof, comprises anti-cancer effects by binding or by inhibiting DPIV, or related enzyme activities, using an inhibitor or ligand of these enzymes. Oral administration of a DPIV inhibitor may be preferable in most circumstances.

The present invention will now be illustrated with reference to the following examples focusing on the anti-cancer-like and anti-metastatic-like action of reduced DPIV-like activity and/or binding in an *in vivo* cancer cell adhesion assay (example 13), and in cancer colonization assays (example 14).

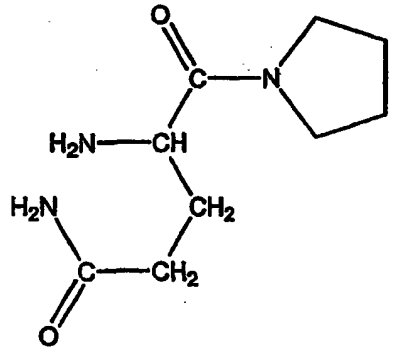
In one illustrative embodiment, the present invention relates to the use of dipeptide compounds and compounds analogous to dipeptide compounds that are formed from an amino acid and a thiazolidine or pyrrolidine group, and salts thereof,

referred to hereinafter as dipeptide compounds. Preferably the amino acid and the thiazolidine or pyrrolidine group are bonded with an amide bond.

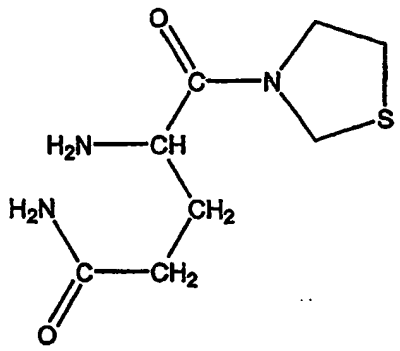
Especially suitable for that purpose according to the invention are dipeptide compounds in which the amino acid is preferably selected from a natural amino acid, such as, for example, leucine, valine, glutamine, glutamic acid, proline, isoleucine, asparagines and aspartic acid.

The dipeptide compounds used according to the invention exhibit at a concentration (of dipeptide compounds) of 10 µM, a reduction in the activity of dipeptidyl peptidase IV or DPIV-analogous enzyme activities of at least 10 %, especially of at least 40 %. Frequently a reduction in activity of at least 60 % or at least 70 % is also required. Preferred effectors may also exhibit a reduction in activity of a maximum of 20 % or 30 %.

Preferred compounds are L-*allo*-isoleucyl thiazolidine, L-*threo*-isoleucyl pyrrolidine and salts thereof, especially the fumaric salts, and L-*allo*-isoleucyl pyrrolidine and salts thereof. Especially preferred compounds are glutaminyl pyrrolidine and glutaminyl thiazolidine of formulas 1 and 2:



(1)



(2)

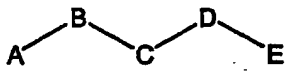
Further preferred compounds are given in Table 1.

The salts of the dipeptide compounds can be present in a molar ratio of dipeptide (-analogous) component to salt component of 1 : 1 or 2 : 1. Such a salt is, for example, (Ile-Thia)$_2$ fumaric acid.

Table 1: Structures of further preferred dipeptide compounds

Effector
H-Asn-pyrrolidine
H-Asn-thiazolidine
H-Asp-pyrrolidine
H-Asp-thiazolidine
H-Asp(NHOH)-pyrrolidine
H-Asp(NHOH)-thiazolidine
H-Glu-pyrrolidine
H-Glu-thiazolidine
H-Glu(NHOH)-pyrrolidine
H-Glu(NHOH)-thiazolidine
H-His-pyrrolidine
H-His-thiazolidine
H-Pro-pyrrolidine
H-Pro-thiazolidine
H-Ile-azididine
H-Ile-pyrrolidine
H-L-*allo*-Ile-thiazolidine
H-Val-pyrrolidine
H-Val-thiazolidine

In another preferred embodiment, the present invention provides the use of peptide compounds of formula 3 useful for competitive modulation of dipeptidyl peptidase IV catalysis:



(3)

wherein

A, B, C, D and E are independently any amino acid moieties including proteinogenic amino acids, non-proteinogenic amino acids, L-amino acids and D-amino acids and wherein E and/or D may be absent.

Further conditions regarding formula (3):

A is an amino acid except a D-amino acid,
B is an amino acid selected from Pro, Ala, Ser, Gly, Hyp, acetidine-(2)-carboxylic acid and pipecolic acid,
C is any amino acid except Pro, Hyp, acetidine-(2)-carboxylic acid, pipecolic acid and except N-alkylated amino acids, e.g. N-methyl valine and sarcosine,
D is any amino acid or missing, and
E is any amino acid or missing,

or:

C is any amino acid except Pro, Hyp, acetidine-(2)-carboxylic acid, pipecolic acid, except N-alkylated amino acids, e.g. N-methyl valine and sarcosine, and except a D-amino-acid;
D is any amino acid selected from Pro, Ala, Ser, Gly, Hyp, acetidine-(2)-carboxylic acid and pipecolic acid, and
E is any amino acid except Pro, Hyp, acetidine-(2)-carboxylic acid, pipecolic acid

and except N-alkylated amino acids, e.g. N-methyl valine and sarcosine.

Examples of amino acids which can be used in the present invention are L and D-amino acids, N-methyl-amino-acids; *allo*- and *threo*-forms of Ile and Thr, which can, e.g. be α-, β- or ω-amino acids, whereof α-amino acids are preferred.

Examples of amino acids throughout the claims and the description are:
aspartic acid (Asp), glutamic acid (Glu), arginine (Arg), lysine (Lys), histidine (His), glycine (Gly), serine (Ser) and cysteine (Cys), threonine (Thr), asparagine (Asn), glutamine (Gln), tyrosine (Tyr), alanine (Ala), proline (Pro), valine (Val), isoleucine (Ile), leucine (Leu), methionine (Met), phenylalanine (Phe), tryptophan (Trp), hydroxyproline (Hyp), beta-alanine (beta-Ala), 2-amino octanoic acid (Aoa), azetidine-(2)-carboxylic acid (Ace), pipecolic acid (Pip), 3-amino propionic, 4-amino butyric and so forth, alpha-aminoisobutyric acid (Aib), sarcosine (Sar), ornithine (Orn), citrulline (Cit), homoarginine (Har), t-butylalanine (*t*-butyl-Ala), t-butylglycine (*t*-butyl-Gly), N-methylisoleucine (N-Melle), phenylglycine (Phg), cyclohexylalanine (Cha), norleucine (Nle), cysteic acid (Cya) and methionine sulfoxide (MSO), Acetyl-Lys, modified amino acids such as phosphoryl-serine (Ser(P)), benzyl-serine (Ser(Bzl)) and phosphoryl-tyrosine (Tyr(P)), 2-aminobutyric acid (Abu), aminoethylcysteine (AECys), carboxymethylcysteine (Cmc), dehydroalanine (Dha), dehydroamino-2-butyric acid (Dhb), carboxyglutaminic acid (Gla), homoserine (Hse), hydroxylysine (Hyl), *cis*-hydroxyproline (*cis*Hyp), *trans*-hydroxyproline (*trans*Hyp), isovaline (Iva), pyroglutamic acid (Pyr), norvaline (Nva), 2-aminobenzoic acid (2-Abz), 3- aminobenzoic acid (3-Abz), 4- aminobenzoic acid (4-Abz), 4-(aminomethyl)benzoic acid (Amb), 4-(aminomethyl)cyclohexanecarboxylic acid (4-Amc), Penicillamine (Pen), 2-Amino-4-cyanobutyric acid (Cba), cycloalkane-carboxylic aicds.
Examples of ω-amino acids are e.g.: 5-Ara (aminoraleric acid), 6-Ahx (aminohexanoic acid), 8-Aoc (aminooctanoic aicd), 9-Anc (aminovanoic aicd), 10-Adc (aminodecanoic acid), 11-Aun (aminoundecanoic acid), 12-Ado (aminododecanoic acid).

Further amino acids are: indanylglycine (Igl), indoline-2-carboxylic acid (Idc), octahydroindole-2-carboxylic acid (Oic), diaminopropionic acid (Dpr), diaminobutyric acid (Dbu), naphtylalanine (1-Nal), (2-Nal), 4-aminophenylalanin (Phe(4-NH_2)), 4-benzoylphenylalanine (Bpa), diphenylalanine (Dip), 4-bromophenylalanine (Phe(4-Br)), 2-chlorophenylalanine (Phe(2-Cl)), 3-chlorophenylalanine (Phe(3-Cl)), 4-chlorophenylalanine (Phe(4-Cl)), 3,4-chlorophenylalanine (Phe (3,4-Cl_2)), 3-fluorophenylalanine (Phe(3-F)), 4- fluorophenylalanine (Phe(4-F)), 3,4-fluorophenylalanine (Phe(3,4-F_2)), pentafluorophenylalanine (Phe(F_5)), 4-guanidinophenylalanine (Phe(4-guanidino)), homophenylalanine (hPhe), 3-jodophenylalanine (Phe(3-J)), 4 jodophenylalanine (Phe(4-J)), 4-methylphenylalanine (Phe(4-Me)), 4-nitrophenylalanine (Phe-4-NO_2)), biphenylalanine (Bip), 4-phosphonomehtylphenylalanine (Pmp), cyclohexyglycine (Ghg), 3-pyridinylalanine (3-Pal), 4-pyridinylalanine (4-Pal), 3,4-dehydroproline (A-Pro), 4-ketoproline (Pro(4-keto)), thioproline (Thz), isonipecotic acid (Inp), 1,2,3,4,-tetrahydroisoquinolin-3-carboxylic acid (Tic), propargylglycine (Pra), 6-hydroxynorleucine (NU(6-OH)), homotyrosine (hTyr), 3-jodotyrosine (Tyr(3-J)), 3,5-dijodotyrosine (Tyr(3,5-J_2)), d-methyl-tyrosine (Tyr(Me)), 3-NO_2-tyrosine (Tyr(3-NO_2)), phosphotyrosine (Tyr(PO_3H_2)), alkylglycine, 1-aminoindane-1-carboxy acid, 2-aminoindane-2-carboxy acid (Aic), 4-amino-methylpyrrol-2-carboxylic acid (Py), 4-amino-pyrrolidine-2-carboxylic acid (Abpc), 2-aminotetraline-2-carboxylic acid (Atc), diaminoacetic acid (Gly(NH_2)), diaminobutyric acid (Dab), 1,3-dihydro-2H-isoinole-carboxylic acid (Disc), homocylcohexylalanin (hCha), homophenylalanin (hPhe oder Hof), *trans*-3-phenyl-azetidine-2-carboxylic acid, 4-phenyl-pyrrolidine-2-carboxylic acid, 5-phenyl-pyrrolidine-2-carboxylic acid, 3-pyridylalanine (3-Pya), 4-pyridylalanine (4-Pya), styrylalanine, tetrahydroisoquinoline-1-carboxylic acid (Tiq), 1,2,3,4-tetrahydronorharmane-3-carboxylic acid (Tpi), ß-(2-thienryl)-alanine (Tha)

Other amino acid substitutions for those encoded in the genetic code can also be included in peptide compounds within the scope of the invention and can be classified within this general scheme.

Proteinogenic amino acids are defined as natural protein-derived α-amino acids. Non-proteinogenic amino acids are defined as all other amino acids, which are not building blocks of common natural proteins.

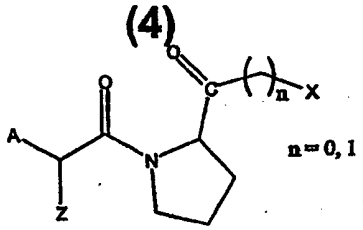
The resulting peptides may be synthesized as the free C-terminal acid or as the C-terminal amide form. The free acid peptides or the amides may be varied by side chain modifications. Such side chain modifications include for instance, but not restricted to, homoserine formation, pyroglutamic acid formation, disulphide bond formation, deamidation of asparagine or glutamine residues, methylation, t-butylation, t-butyloxycarbonylation, 4-methylbenzylation, thioanysilation, thiocresylation, bencyloxymethylation, 4-nitrophenylation, bencyloxycarbonylation, 2-nitrobencoylation, 2-nitrosulphenylation, 4-toluenesulphonylation, pentafluorophenylation, diphenylmethylation, 2-chlorobenzyloxycarbonylation, 2,4,5-trichlorophenylation, 2-bromobenzyloxycarbonylation, 9-fluorenylmethyloxycarbonylation, triphenylmethylation, 2,2,5,7,8,-pentamethylchroman-6-sulphonylation, hydroxylation, oxidation of methionine, formylation, acetylation, anisylation, bencylation, bencoylation, trifluoroacetylation, carboxylation of aspartic acid or glutamic acid, phosphorylation, sulphation, cysteinylation, glycolysation with pentoses, deoxyhexoses, hexosamines, hexoses or N-acetylhexosamines, farnesylation, myristolysation, biotinylation, palmitoylation, stearoylation, geranylgeranylation, glutathionylation, 5'-adenosylation, ADP-ribosylation, modification with N-glycolylneuraminic acid, N-acetylneuraminic acid, pyridoxal phosphate, lipoic acid, 4'-phosphopantetheine, or N-hydroxysuccinimide.

In the compounds of formula (3), the amino acid moieties A, B, C, D, and E are respectively attached to the adjacent moiety by amide bonds in a usual manner according to standard nomenclature so that the amino-terminus (N-terminus) of the amino acids (peptide) is drawn on the left and the carboxyl-terminus of the amino acids (peptide) is drawn on the right. (C-terminus)

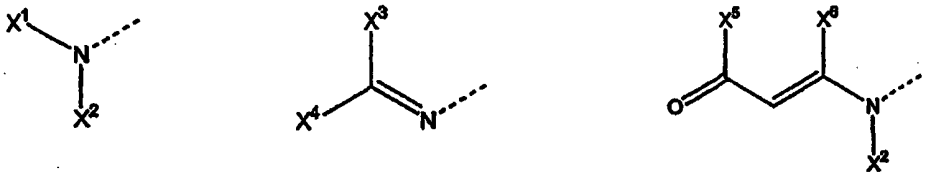
Until the present invention by Applicants, known peptide substrates of the proline-specific serine protease dipeptidyl peptidase IV *in vitro* are the tripeptides Diprotin A (Ile-Pro-Ile), Diprotin B (Val-Pro-Leu) and Diprotin C (Val-Pro-Ile). Applicants have unexpectedly discovered that the compounds disclosed here act as substrates of dipeptidyl peptidase IV *in vivo* in a mammal and, in pharmacological doses, inhibit the physiological turnover of endogenous substrates by competitive catalysis.

Particularly preferred compounds of the present invention that are useful as modulators of dipeptidyl peptidase IV and DPIV – like enzymes include those compounds which show K_i-values for DPIV-binding, effectively in DPIV-inhibition *in vivo* after i.v. and/or p.o. administration to Wistar rats.

Further preferred compounds are peptidylketones of formula 4:



wherein
A is selected from :



X^1 is H or an acyl or oxycarbonyl group incl. all amino acids and peptide residues,

X^2 is H, $-(CH)_n$-NH-C_5H_3N-Y with n=2-4 or C_5H_3N-Y (a divalent pyridyl residue) and Y is selected from H, Br, Cl, I, NO_2 or CN,

X^3 is H or a phenyl or pyridyl residue, unsubstituted or substituted with one, two or more alkyl, alkoxy, halogen, nitro, cyano or carboxy residues,

X^4 is H or a phenyl or pyridyl residue, unsubstituted or substituted with one, two or more alkyl, alkoxy, halogen, nitro, cyano or carboxy residues,

X^5 is H or an alkyl, alkoxy or phenyl residue,

X^6 is H or an alkyl residue.

for n = 1

X is selected from: H, OR^2, SR^2, NR^2R^3, $N^+R^2R^3R^4$, wherein:

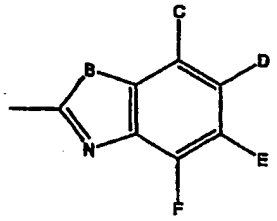
R^2 stands for acyl residues, which are unsubstituted or substituted with one, two or more alkyl, cycloalkyl, aryl or heteroaryl residues, or for all amino acids and peptidic residues, or alkyl residues, which are unsubstituted or substituted with one, two or more alkyl, cycloalkyl, aryl and heteroaryl residues,

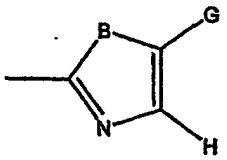
R^3 stands for alkyl and acyl functions, wherein R^2 and R^3 may be part of one or more ring structures of saturated and unsaturated carbocyclic or heterocyclic structures,

R^4 stands for alkyl residues, wherein R^2 and R^4 or R^3 and R^4 may be part of one or more ring structures of saturated and unsaturated carbocyclic or heterocyclic structures,

for n = 0

X is selected from:





wherein

B stands for: O, S, NR^5, wherein R^5 is H, an alkyliden or acyl,

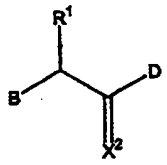
C, D, E, F, G, H are independently selected from unsubstituted and substituted alkyl, oxyalkyl, thioalkyl, aminoalkyl, carbonylalkyl, acyl, carbamoyl, aryl and heteroaryl residues; and

for n= 0 and n=1

Z is selected from H, or a branched or single chain alkyl residue from C_1-C_9 or a branched or single chain alkenyl residue from C_2-C_9, a cycloalkyl residue from C_3-C_8, a cycloalkenyl residue from C_5-C_7, an aryl- or heteroaryl residue, or a side chain selected from all side chains of all natural amino acids or derivatives thereof.

Further, according to the present invention compounds of formulas 5, 6, 7,8, 9, 10 and 11, including all stereoisomers and pharmaceutical acceptable salts thereof are disclosed and can be used:

(5)



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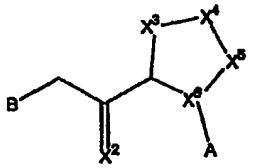

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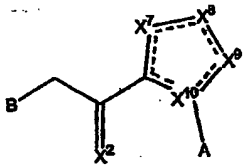

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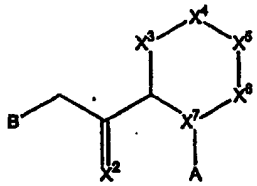

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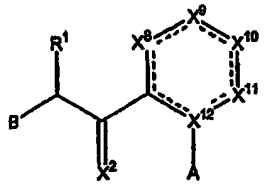

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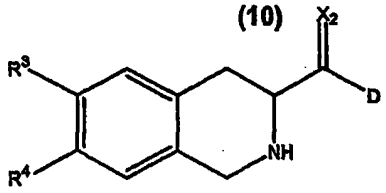

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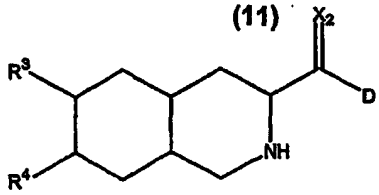


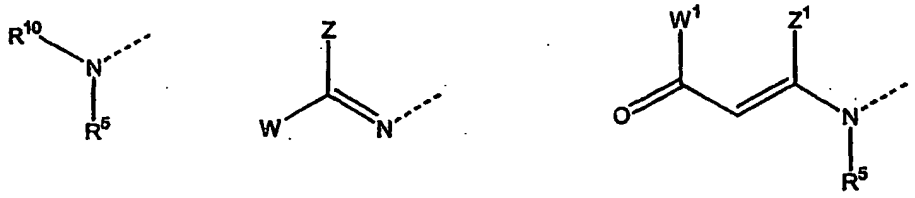
wherein:

R^1 is H, a branched or linear C_1-C_9 alkyl residue, a branched or linear C_2-C_9 alkenyl residue, a C_3-C_8 cycloalkyl- , C_5-C_7 cycloalkenyl-, aryl- or heteroaryl residue or a side chain of a natural amino acid or a derivative thereof,

R^3 and R^4 are selected from H, hydroxy, alkyl, alkoxy, aryloxy, nitro, cyano or halogen,

A is H or an isoster of a carbonic acid, like a functional group selected from CN, SO_3H, CONHOH, $PO_3R^5R^6$, tetrazole, amide, ester, anhydride, thiazole and imidazole,

B is selected from:



wherein :

R^5 is H, $-(CH)_n$-NH-C_5H_3N-Y with n=2-4 and C_5H_3N-Y (a divalent pyridyl residue) with Y = H, Br, Cl, I, NO_2 or CN ,

R^{10} is H, an acyl, oxycarbonyl or a amino acid residue ,

W is H or a phenyl or pyridyl residue, unsubstituted or substituted with one, two or more alkyl, alkoxy, halogen, nitro, cyano or carboxy residues,

W^1 is H, an alkyl, alkoxy or phenyl residue,

Z is H or a phenyl or pyridyl residue, unsubstituted or substituted with one, two or more alkyl, alkoxy, halogen, nitro, cyano or carboxy residues,

Z^1 is H or an alkyl residue,

D is a cyclic C_4-C_7 alkyl, C_4-C_7 alkenyl residue which can be unsubstituted or substituted with one, two or more alkyl groups or a cyclic 4-7-membered heteroalkyl or a cyclic 4-7-membered heteroalkenyl residue,

X^2 is O, NR^6, $N^+(R^7)_2$, or S,

X^3 to X^{12} are independently selected from CH_2, CR^8R^9, NR^6, $N^+(R^7)_2$, O, S, SO and SO_2, including all saturated and unsaturated structures,

R^6, R^7, R^8, R^9 are independently selected from H, a branched or linear C_1-C_8 alkyl residue, a branched or linear C_2-C_8 alkenyl residue, a C_3-C_8 cycloalkyl residue, a C_5-C_7 cycloalkenyl residue, an aryl or heteroaryl residue,

with the following provisions:

Formula 6: X^6 is CH if A is not H,
Formula 7: X^{10} is C if A is not H,
Formula 8: X^7 is CH if A is not H,
Formula 9: X^{12} is C if A is not H.

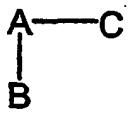
Throughout the description and the claims the expression "acyl" can denote a C_{1-20} acyl residue, preferably a C_{1-8} acyl residue and especially preferred a C_{1-4} acyl residue, "cycloalkyl" can denote a C_{3-12} cycloalkyl residue, preferably a C_4, C_5 or C_6 cycloalkyl residue, "carbocyclic" can denote a C_{3-12} carbocyclic residue, preferably a C_4, C_5 or C_6 carbocyclic residue. "Heteroaryl" is defined as an aryl residue, wherein 1 to

4, preferably 1, 2 or 3 ring atoms are replaced by heteroatoms like N, S or O. "Heterocyclic" is defined as a cycloalkyl residue, wherein 1, 2 or 3 ring atoms are replaced by heteroatoms like N, S or O. "Peptides" are selected from dipeptides to decapeptides, preferred are dipeptides, tripeptides, tetrapeptides and pentapeptides. The amino acids for the formation of the "peptides" can be selected from the those listed above.

Because of the wide distribution of the protein in the body and the wide variety of mechanisms involving DPIV, DPIV-activity and DPIV-related proteins, systemic therapy (enteral or parenteral administration) with DPIV-inhibitors can result in a series of undesirable side-effects.

The problem to be solved was moreover, to provide compounds that can be used for targeted influencing of locally limited pathophysiological and physiological processes. The problem of the invention especially consists in obtaining locally limited inhibition of DPIV or DPIV-analogous activity for the purpose of targeted intervention in the regulation of the activity of locally active substrates.

This problem is solved according to the invention by compounds of the general formula (12)



(12)

wherein

A is an amino acid having at least one functional group in the side chain,

B is a chemical compound covalently bound to at least one functional group of the side chain of A,

C is a thiazolidine, pyrrolidine, cyanopyrrolidine, hydroxyproline, dehydroproline or piperidine group amide-bonded to A.

The compounds can be used for reducing immune, autoimmune or central nervous system related disorders.

In accordance with a preferred embodiment of the invention, pharmaceutical compositions are used comprising at least one compound of the general formula (12) and at least one customary adjuvant appropriate for the site of action.

Preferably A is an α-amino acid, especially a natural α-amino acid having one, two or more functional groups in the side chain, preferably threonine, tyrosine, serine, arginine, lysine, aspartic acid, glutamic acid or cysteine.

Preferably B is an oligopeptide having a chain length of up to 20 amino acids, a polyethylene glycol having a molar mass of up to 20 000 g/mol, an optionally substituted organic amine, amide, alcohol, acid or aromatic compound having from 8 to 50 C atoms.

Throughout the description and the claims the expression "alkyl" can denote a C_{1-50} alkyl group, preferably a C_{6-30} alkyl group, especially a C_{8-12} alkyl group; for example, an alkyl group may be a methyl, ethyl, propyl, isopropyl or butyl group. The expression "alk", for example in the expression "alkoxy", and the expression "alkan", for example in the expression "alkanoyl", are defined as for "alkyl"; aromatic compounds are preferably substituted or optionally unsubstituted phenyl, benzyl, naphthyl, biphenyl or anthracene groups, which preferably have at least 8 C atoms; the expression "alkenyl" can denote a C_{2-10} alkenyl group, preferably a C_{2-6} alkenyl group, which has the double bond(s) at any desired location and may be substituted or unsubstituted; the expression "alkynyl" can denote a C_{2-10} alkynyl group, preferably a C_{2-6} alkynyl group, which has the triple bond(s) at any desired location and may be

substituted or unsubstituted; the expression "substituted" or substituent can denote any desired substitution by one or more, preferably one or two, alkyl, alkenyl, alkynyl, mono- or multi-valent acyl, alkanoyl, alkoxyalkanoyl or alkoxyalkyl groups; the aforementioned substituents may in turn have one or more (but preferably zero) alkyl, alkenyl, alkynyl, mono- or multi-valent acyl, alkanoyl, alkoxyalkanoyl or alkoxyalkyl groups as side groups; organic amines, amides, alcohols or acids, each having from 8 to 50 C atoms, preferably from 10 to 20 C atoms, can have the formulae $(alkyl)_2N$- or alkyl-NH-, -CO-N$(alkyl)_2$ or -CO-NH(alkyl), -alkyl-OH or -alkyl-COOH.

Despite an extended side chain function, the compounds of formula (12) can still bind to the active centre of the enzyme dipeptidyl peptidase IV and analogous enzymes but are no longer actively transported by the peptide transporter PepT1. The resulting reduced or greatly restricted transportability of the compounds according to the invention leads, in ideal manner, to local or site directed inhibition of DPIV and DPIV-like enzyme activity.

The compounds of formula (12) or the other compounds and prodrugs used in accordance with the invention can be present or used, respectively, in the form of racemates or in the form of enantiomerically pure compounds, preferably in the L-threo or L-allo form with respect to part A of formula (12).

By extending/expanding the side chain modifications, for example beyond a number of seven carbon atoms, it is accordingly possible to obtain a dramatic reduction in transportability (see Example 12). The Examples in Table 12.1 clearly show that, with increasing spatial size of the side chains, there is a reduction in the transportability of the substances. By spatially and sterically expanding the side chains, for example beyond the atom group size of a monosubstituted phenyl radical, hydroxylamine radical or amino acid residue, it is possible according to the invention to modify or suppress the transportability of the target substances.

According to the present invention, the compounds of formula (12) inhibit DPIV or DPIV-like enzyme activity in the body of a mammal in a site specific manner. It is accordingly possible to influence local physiological and pathophysiological conditions (inflammation, psoriasis, arthritis, autoimmune diseases, allergies, cancer, metastasis) effectively and with dramatically reduced side-effects.

Preferred compounds of formula (12) are compounds, wherein the oligopeptides have chain lengths of from 3 to 15, especially from 4 to 10, amino acids, and/or the polyethylene glycols have molar masses of at least 250 g/mol, preferably of at least 1500 g/mol and up to 15 000 g/mol, and/or the optionally substituted organic amines, amides, alcohols, acids or aromatic compounds have at least 12 C atoms and preferably up to 30 C atoms.

The compounds of the present invention can be converted into and used as acid addition salts, especially pharmaceutically acceptable acid addition salts. The pharmaceutically acceptable salt generally takes a form in which an amino acids basic side chain is protonated with an inorganic or organic acid. Representative organic or inorganic acids include hydrochloric, hydrobromic, perchloric, sulfuric, nitric, phosphoric, acetic, propionic, glycolic, lactic, succinic, maleic, fumaric, malic, tartaric, citric, benzoic, mandelic, methanesulfonic, hydroxyethanesulfonic, benzenesulfonic, oxalic, pamoic, 2-naphthalenesulfonic, p-toulenesulfonic, cyclohexanesulfamic, salicylic, saccharinic or trifluoroacetic acid. All pharmaceutically acceptable acid addition salt forms of the compounds of formulas (1) to (12) are intended to be embraced by the scope of this invention.

In view of the close relationship between the free compounds and the compounds in the form of their salts, whenever a compound is referred to in this context, a corresponding salt is also intended, provided such is possible or appropriate under the circumstances.

The present invention further includes within its scope prodrugs of the compounds of this invention. In general, such prodrugs will be functional derivatives of the compounds which are readily convertible *in vivo* into the desired therapeutically active compound. Thus, in these cases, the use of the present invention shall encompass the treatment of the various disorders described with prodrug versions of one or more of the claimed compounds, which convert to the above specified compound *in vivo* after administration to the subject. Conventional procedures for the selection and preparation of suitable prodrug derivatives are described, for example, in "Design of Prodrugs", ed. H. Bundgaard, Elsevier, 1985 and the patent applications DE 198 28 113 and DE 198 28 114, which are fully incorporated herein by reference.

Where the compounds or prodrugs according to this invention have at least one chiral center, they may accordingly exist as enantiomers. Where the compounds or prodrugs possess two or more chiral centers, they may additionally exist as diastereomers. It is to be understood that all such isomers and mixtures thereof are encompassed within the scope of the present invention. Furthermore, some of the crystalline forms of the compounds or prodrugs may exist as polymorphs and as such are intended to be included in the present invention. In addition, some of the compounds may form solvates with water (i.e. hydrates) or common organic solvents, and such solvates are also intended to be encompassed within the scope of this invention.

The compounds, including their salts, can also be obtained in the form of their hydrates, or include other solvents used for their crystallization.

As indicated above, the compounds and prodrugs of the present invention, and their corresponding pharmaceutically acceptable acid addition salt forms, are useful in inhibiting DPIV and DPIV – like enzyme activity. The ability of the compounds and prodrugs of the present invention, and their corresponding pharmaceutically acceptable acid addition salt forms to inhibit DPIV and DPIV – like enzyme activity may

be demonstrated employing the DPIV activity assay for determination of the K_i-values and the IC_{50}-values *in vitro*, as described in examples 7 and 8.

The ability of the compounds of the present invention, and their corresponding pharmaceutically acceptable acid addition salt forms to inhibit DPIV *in vivo* may be demonstrated by oral or intravasal administration to Wistar rats, as described in example 11. The compounds of the present invention inhibit DPIV activity *in vivo* after both, oral and intravasal administration to Wistar rats.

DPIV is present in a wide variety of mammalian organs and tissues e.g. the intestinal brush-border (Gutschmidt S. et al., "In situ" - measurements of protein contents in the brush border region along rat jejunal villi and their correlations with four enzyme activities. Histochemistry 1981, 72 (3), 467-79), exocrine epithelia, hepatocytes, renal tubuli, endothelia, myofibroblasts (Feller A.C. et al., A monoclonal antibody detecting dipeptidylpeptidase IV in human tissue. Virchows Arch. A. Pathol. Anat. Histopathol. 1986; 409 (2):263-73), nerve cells, lateral membranes of certain surface epithelia, e.g. Fallopian tube, uterus and vesicular gland, in the luminal cytoplasm of e.g., vesicular gland epithelium, and in mucous cells of Brunner's gland (Hartel S. et al., Dipeptidyl peptidase (DPP) IV in rat organs. Comparison of immunohistochemistry and activity histochemistry. Histochemistry 1988; 89 (2): 151-61), reproductive organs, e.g. cauda epididymis and ampulla, seminal vesicles and their secretions (Agrawal & Vanha-Perttula, Dipeptidyl peptidases in bovine reproductive organs and secretions. Int. J. Androl. 1986, 9 (6): 435-52). In human serum, two molecular forms of dipeptidyl peptidase are present (Krepela E. et al., Demonstration of two molecular forms of dipeptidyl peptidase IV in normal human serum. Physiol. Bohemoslov. 1983, 32 (6): 486-96). The serum high molecular weight form of DPIV is expressed on the surface of activated T cells (Duke-Cohan J.S. et al., Serum high molecular weight dipeptidyl peptidase IV (CD26) is similar to a novel antigen DPPT-L released from activated T cells. J. Immunol. 1996, 156 (5): 1714-21).

The compounds and prodrugs of the present invention, and their corresponding pharmaceutically acceptable acid addition salt forms are able to inhibit DPIV *in vivo*. In one embodiment of the present invention, all molecular forms, homologues and epitopes of DPIV from all mammalian tissues and organs, also of those, which are undiscovered yet, are intended to be embraced by the scope of this invention.

Among the rare group of proline-specific proteases, DPIV was originally believed to be the only membrane-bound enzyme specific for proline as the penultimate residue at the amino-terminus of the polypeptide chain. However, other molecules, even structurally non-homologous with the DPIV but bearing corresponding enzyme activity, have been identified recently. DPIV-like enzymes, which are identified so far, are e.g. fibroblast activation protein α, dipeptidyl peptidase IV β, dipeptidyl aminopeptidase-like protein, N-acetylated α-linked acidic dipeptidase, quiescent cell proline dipeptidase, dipeptidyl peptidase II, attractin and dipeptidyl peptidase IV related protein (DPP 8), and are described in the review article by Sedo & Malik (Sedo & Malik, Dipeptidyl peptidase IV-like molecules: homologous proteins or homologous activities? Biochimica et Biophysica Acta 2001, 36506: 1-10). Further DPIV like enzymes are disclosed in WO 01/19866, WO 02/04610 and WO 02/34900. WO 01/19866 discloses novel human dipeptidyl aminopeptidase (DPP8) with structural und functional similarities to DPIV and fibroblast activation protein (FAP). The dipeptidyl peptidase IV-like enzyme of WO 02/04610 is well known in the art. In the Gene Bank data base, this enzyme is registered as KIAA1492. In another preferred embodiment of the present invention, all molecular forms, homologues and epitopes of proteins comprising DPIV-like enzyme activity, from all mammalian tissues and organs, also of those, which are undiscovered yet, are intended to be embraced by the scope of this invention.

The ability of the compounds and prodrugs of the present invention, and their corresponding pharmaceutically acceptable acid addition salt forms to inhibit DPIV-like enzymes may be demonstrated employing an enzyme activity assay for determination of the K_i-values *in vitro* as described in example 9. The K_i-values of the compounds of

the present invention against porcine dipeptidyl peptidase II were exemplary determined as K_i = $8.52*10^{-5}$ M ± $6.33*10^{-6}$ M for glutaminyl pyrrolidine and K_i = $1.07*10^{-5}$ M ± $3.81*10^{-7}$ M for glutaminyl thiazolidine.

In another embodiment, the compounds and prodrugs of the present invention, and their corresponding pharmaceutically acceptable acid addition salt forms have only low, if no inhibitory activity against non-DPIV and non-DPIV – like proline specific enzymes. As described in example 10, with glutaminyl thiazolidine and glutaminyl pyrrolidine exemplarily, no inhibition of dipeptidyl peptidase I and prolyl oligopeptidase was found. Against prolidase, both compounds showed a marked lower efficacy compared to DPIV. The IC 50-values against prolidase were determined as IC 50 > 3mM for glutaminyl thiazolidine and as IC 50 = $3.4*10^{-4}$M ± $5.63*10^{-5}$ for glutaminyl pyrrolidine.

The present invention provides a method of preventing or treating a condition mediated by modulation of the DPIV or DPIV – like enzyme activity in a subject in need thereof which comprises administering any of the compounds of the present invention or pharmaceutical compositions thereof in a quantity and dosing regimen therapeutically effective to treat the condition. Additionally, the present invention includes the use of the compounds and prodrugs of this invention, and their corresponding pharmaceutically acceptable acid addition salt forms, for the preparation of a medicament for the prevention or treatment of a condition mediated by modulation of the DPIV activity in a subject. The compound may be administered to a patient by any conventional route of administration, including, but not limited to, intravenous, oral, subcutaneous, intramuscular, intradermal, parenteral and combinations thereof.

In a further illustrative embodiment, the present invention provides formulations for the compounds of formulas 1 to 12, and their corresponding pharmaceutically acceptable prodrugs and acid addition salt forms, in pharmaceutical compositions.

The term "subject" as used herein, refers to an animal, preferably a mammal, most preferably a human, who has been the object of treatment, observation or experiment.

The term "therapeutically effective amount" as used herein, means that amount of active compound or pharmaceutical agent that elicits the biological or medicinal response in a tissue system, animal or human, being sought by a researcher, veterinarian, medical doctor or other clinician, which includes alleviation of the symptoms of the disease or disorder being treated.

As used herein, the term "composition" is intended to encompass a product comprising the claimed compounds in the therapeutically effective amounts, as well as any product which results, directly or indirectly, from combinations of the claimed compounds.

To prepare the pharmaceutical compositions used in this invention, one or more compounds of formulas 1 to 12, or their corresponding pharmaceutically acceptable prodrugs or acid addition salt forms, as the active ingredient, is intimately admixed with a pharmaceutical carrier according to conventional pharmaceutical compounding techniques, which carrier may take a wide variety of forms depending of the form of preparation desired for administration, e.g., oral or parenteral such as intramuscular. In preparing the compositions in oral dosage form, any of the usual pharmaceutical media may be employed. Thus, for liquid oral preparations, such as for example, suspensions, elixirs and solutions, suitable carriers and additives may advantageously include water, glycols, oils, alcohols, flavoring agents, preservatives, coloring agents and the like; for solid oral preparations such as, for example, powders, capsules, gelcaps and tablets, suitable carriers and additives include starches, sugars, diluents, granulating agents, lubricants, binders, disintegrating agents and the like. Because of their ease in administration, tablets and capsules represent the most advantageous oral dosage unit form, in which case solid pharmaceutical carriers are employed. If desired, tablets may be sugar coated or enteric coated by standard techniques. For

parenterals the carrier will usually comprise sterile water, through other ingredients, for example, for purposes such as aiding solubility or for preservation, may be included.

Injectable suspensions may also be prepared, in which case appropriate liquid carriers, suspending agents and the like may be employed. The pharmaceutical compositions herein will contain, per dosage unit, e.g., tablet, capsule, powder, injection, teaspoonful and the like, an amount of the active ingredient necessary to deliver an effective dose as described above. The pharmaceutical compositions herein will contain, per dosage unit, e.g., tablet, capsule, powder, injection, suppository, teaspoonful and the like, of from about 0.01 mg to about 1000 mg (preferably about 5 to about 500 mg) and may be given at a dosage of from about 0.1 to about 300 mg/kg bodyweight per day (preferably 1 to 50 mg/kg per day). The dosages, however, may be varied depending upon the requirement of the patients, the severity of the condition being treated and the compound being employed. The use of either daily administration or post-periodic dosing may be employed. Typically the dosage will be regulated by the physician based on the characteristics of the patient, his/her condition and the therapeutic effect desired.

Preferably these compositions are in unit dosage forms from such as tablets, pills, capsules, powders, granules, sterile parenteral solutions or suspensions, metered aerosol or liquid sprays, drops, ampoules, autoinjector devices or suppositories; for oral parenteral, intranasal, sublingual or rectal administration, or for administration by inhalation or insufflation. Alternatively, the composition may be presented in a form suitable for once-weekly or once-monthly administration; for example, an insoluble salt of the active compound, such as the decanoate salt, may be adapted to provide a depot preparation for intramuscular injection. For preparing solid compositions such as tablets, the principal active ingredient is ideally mixed with a pharmaceutical carrier, e.g. conventional tableting ingredients such as corn starch, lactose, sucrose, sorbitol, talc, stearic acid, magnesium stearate, dicalcium phosphate or gums, and other pharmaceutical diluents, e.g. water, to form a solid preformulation composition containing a homogeneous mixture of a compound of the present invention, or a

pharmaceutically acceptable salt thereof. When referring to these preformulation compositions as homogeneous, it is meant that the active ingredient is ideally dispersed evenly throughout the composition so that the composition may be readily subdivided into equally effective dosage forms such as tablets, pills and capsules. This solid preformulation composition may then be subdivided into unit dosage forms of the type described above containing from about 0.01 to about 1000 mg, preferably from about 5 to about 500 mg of the active ingredient of the present invention.

The tablets or pills of the novel composition can be advantageously coated or otherwise compounded to provide a dosage form affording the advantage of prolonged action. For example, the tablet or pill can comprise an inner dosage and an outer dosage component, the latter being in the form of an envelope over the former. The two components can be separated by an enteric layer which serves to resist disintegration in the stomach and permits the inner component to pass intact into the duodenum or to be delayed in release. A variety of materials can be used for such enteric layers or coatings, such materials including a number of polymeric acids with such materials as shellac, cetyl alcohol and cellulose acetate.

This liquid forms in which the novel compositions of the present invention may be advantageously incorporated for administration orally or by injection include, aqueous solutions, suitably flavored syrups, aqueous or oil suspensions, and flavored emulsions with edible oils such as cottonseed oil, sesame oil, coconut oil or peanut oil, as well as elixirs and similar pharmaceutical vehicles. Suitable dispersing or suspending agents for aqueous suspensions include synthetic and natural gums such as tragacanth, acacia, alginate, dextran, sodium carboxymethylcellulose, methylcellulose, polyvinylpyrrolidone or gelatin.

Where the processes for the preparation of the compounds according to the invention give rise to a mixture of stereoisomers, these isomers may be separated by conventional techniques such as preparative chromatography. The compounds may be prepared in racemic form, or individual enantiomers may be prepared either by

enantiospecific synthesis or by resolution. The compounds may, for example, be resolved into their components enantiomers by standard techniques, such as the formation of diastereomeric pairs by salt formation with an optically active acid, such as (-)-di-p-toluoyl-d-tartaric acid and/or (+)-di-p-toluoyl-l-tartaric acid followed by fractional crystallization and regeneration of the free base. The compounds may also resolved by formation of diastereomeric esters or amides, followed by chromatographic separation and removal of the chiral auxiliary. Alternatively, the compounds may be resolved using a chiral HPLC column.

During any of the processes for preparation of the compounds of the present invention, it may be necessary and/or desirable to protect sensitive or reactive groups on any of the molecules concerned. This may be achieved by means of conventional protecting groups, such as those described in Protective Groups in Organic Chemistry, ed. J.F.W. McOmie, Plenum Press, 1973; and T.W. Greene & P.G.M. Wuts, Protective Groups in Organic Synthesis, John Wiley & Sons, 1991, fully incorporated herein by reference. The protecting groups may be removed at a convenient subsequent stage using methods known from the art.

The method of treating conditions modulated by dipeptidyl peptidase IV and DPIV - like enzymes described in the present invention may also be carried out using a pharmaceutical composition comprising one or more of the compounds as defined herein and a pharmaceutically acceptable carrier. The pharmaceutical composition may contain from about 0.01 mg to 1000 mg, preferably about 5 to about 500 mg, of the compound(s), and may be constituted into any form suitable for the mode of administration selected. Carriers include necessary and inert pharmaceutical excipients, including, but not limited to, binders, suspending agents, lubricants, flavorants, sweeteners, preservatives, dyes, and coatings. Compositions suitable for oral administration include solid forms, such as pills, tablets, caplets, capsules (each including immediate release, timed release and sustained release formulations), granules, and powders, and liquid forms, such as solutions, syrups, elixirs, emulsions,

and suspensions. Forms useful for parenteral administration include sterile solutions, emulsions and suspensions.

Advantageously, compounds of the present invention may be administered in a single daily dose, or the total daily dosage may be administered in divided doses of two, three or four times daily. Furthermore, compounds of the present invention can be administered in intranasal form via topical use of suitable intranasal vehicles, or via transdermal skin patches well known to those of ordinary skill in that art. To be administered in the form of transdermal delivery system, the dosage administration will, of course, be continuous rather than intermittent throughout the dosage regimen and dosage strength will need to be accordingly modified to obtain the desired therapeutic effects.

More preferably, for oral administration in the form of a tablet or capsule, the active drug component can be combined with an oral, non-toxic pharmaceutically acceptable inert carrier such as ethanol, glycerol, water and the like. Moreover, when desired or necessary, suitable binders; lubricants, disintegrating agents and coloring agents can also be incorporated into the mixture. Suitable binders include, without limitation, starch, gelatin, natural sugars such as glucose or betalactose, corn sweeteners, natural and synthetic gums such as acacia, tragacanth or sodium oleate, sodium stearate, magnesium stearate, sodium benzoate, sodium acetate, sodium chloride and the like. Disintegrators include, without limitation, starch, methyl cellulose, agar, bentonite, xanthan gum and other compounds known within the art.

The liquid forms are suitable in flavored suspending or dispersing agents such as the synthetic and natural gums, for example, tragacanth, acacia, methyl-cellulose and the like. For parenteral administration, sterile suspensions and solutions are desired. Isotonic preparations which generally contain suitable preservatives are employed when intravenous administration is desired.

The compound of the present invention can also be administered in the form of liposome delivery systems, such as small unilamellar vesicles, large unilamellar vesicles, and multilamellar vesicles. Liposomes can be formed from a variety of phospholipids, such as cholesterol, stearylamine or phosphatidylcholines using processes well described in the art.

Compounds of the present invention may also be coupled with soluble polymers as targetable drug carriers. Such polymers can include polyvinylpyrrolidone, pyran copolymer, polyhydroxypropylmethacrylamidephenol, polyhydroxyethylaspartamide-phenol, or polyethyl eneoxidepolylysine substituted with palmitoyl residue. Furthermore, compounds of the present invention may be coupled to a class of biodegradable polymers useful in achieving controlled release of a drug, for example, polyactic acid, polyepsilon caprolactone, polyhydroxy butyeric acid, polyorthoesters, polyacetals, polydihydropyrans, polycyanoacrylates and cross-linked or amphipathic block copolymers of hydrogels.

Compounds of this invention may be administered in any of the foregoing compositions and according to dosage regimens established in the art whenever treatment of the addressed disorders is required.

The daily dosage of the products may be varied over a wide range from 0.01 to 1.000 mg per adult human per day. For oral administration, the compositions are preferably provided in the form of tablets containing, 0.01, 0.05, 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, 25.0, 50.0, 100, 150, 200, 250, 500 and 1000 milligrams of the active ingredient for the symptomatic adjustment of the dosage to the patient to be treated. An effective amount of the drug is ordinarily supplied at a dosage level of from about 0.1 mg/kg to about 300 mg/kg of body weight per day. Preferably, the range is from about 1 to about 50 mg/kg of body weight per day. The compounds may be administered on a regimen of 1 to 4 times per day.

Optimal dosages to be administered may be readily determined by those skilled in the art, and will vary with the particular compound used, the mode of administration, the strength of the preparation, bioavailability due to the mode of administration, and the advancement of disease condition. In addition, factors associated with the particular patient being treated, including patient age, weight, diet and time of administration, should generally be considered in adjusting dosages.

The compounds or compositions of the present invention may be taken before a meal, while taking a meal or after a meal.

When taken while eating, the compounds or compositions of the present invention can be mixed into the meal or taken in a separate dosage form as described above.

Examples

Example 1: Synthesis of dipeptide compounds

1.1 General synthesis of isoleucyl thiazolidine salt

The Boc-protected amino acid BOC-Ile-OH is placed in ethyl acetate and the batch is cooled to about – 5°C. N-Methylmorpholine is added dropwise, pivalic acid chloride (on a laboratory scale) or neohexanoyl chloride (on a pilot-plant scale) is added dropwise at constant temperature. The reaction is stirred for a few minutes for activation. N-Methylmorpholine (laboratory scale) and thiazolidine hydrochloride (laboratory scale) are added dropwise in succession, thiazolidine (pilot-plant scale) is added. Working-up in the laboratory is effected in conventional manner using salt solutions, on a pilot-plant scale the batch is purified with NaOH and CH_3COOH solutions.

The removal of the BOC protecting group is carried out using HCl/dioxane (laboratory scale) or H_2SO_4 (pilot-plant scale). In the laboratory the hydrochloride is crystallised from EtOH/ether.

On a pilot-plant scale the free amine is prepared by the addition of $NaOH/NH_3$. Fumaric acid is dissolved in hot ethanol, the free amine is added dropwise, and (Ile-Thia)^2 fumarate (M = 520.71 gmol^{-1}) precipitates. The analysis of isomers and enantiomers is carried out by electrophoresis.

1.2 Synthesis of glutaminyl pyrrolidine free base

Acylation:

N-Benzyl-oxycarbonylglutamine (2.02 g, 7.21 mmol) was dissolved in 35 ml THF and brought to –15°C. Into that mixture CAIBE (isobutylchloroformiate) (0.937 ml, 7.21 mmol) and 4-methylmorpholine (0.795 ml, 7.21 mmol) where added and the solution was stirred for 15 min. The formation of the mixed anhydride was checked by TLC (eluent: $CHCl_3$/MeOH: 9/1). After warming to –10°C pyrrolidine (0.596 ml, 7.21 mmol) was added. The mixture was brought to room temperature and stirred overnight.

Workup:

The sediment formed was filtered off and the solvent was evaporated. The resulting oil was taken up in ethylacetate (20 ml) and washed with a saturated solution of sodiumhydrogensulfate followed by a saturated solution of sodiumbicarbonate, water and brine. The organic layer was separated, dried and evaporated. The resulting product was checked for purity by TLC (eluent: $CHCl_3$/MeOH: 9/1)
Yield: 1.18 g, waxy solid

Cleavage:

1.18 g of the resulting solid Z-protected compound was dissolved in 40 ml absolute ethanol. Into the solution ca. 20 mg Pd on charcoal (10%, FLUKA) was

added and the suspension was shaken under a hydrogen atmosphere for 3h. The progress of the reaction was monitored by TLC (eluent: $CHCl_3$/MeOH: 9/1). After completion of the reaction the was removed to provide the free base.
Yield: 99%
The purity was checked by means of TLC: n-butanole/AcOH/water/ethylacetate: 1/1/1/1, $R_f = 0.4$. The identity of the reaction product was checked by NMR analysis.

1.3 Synthesis of glutaminyl thiazolidine hydrochloride

Acylation:

N-t-Butyl-oxycarbonylglutamine (2.0 g, 8.12 mmol) was dissolved in 5ml THF and brought to –15°C. Into that mixture CAIBE (isobutylchloroformiate) (1,06 ml, 8.12 mmol) and 4-methylmorpholine (0.895 ml, 8.12 mmol) where added and the solution was stirred for 15 min. The formation of the mixed anhydride was checked by TLC (eluent: $CHCl_3$/MeOH: 9/1). After warming to –10°C another equivalent 4-methylmorpholine (0.895 ml, 8.12 mmol) and thiazolidinehydrochloride (1.02 g, 8.12 mmol was added. The mixture was brought to room temperature and stirred overnight.

Workup:

The sediment formed was filtered off and the solvent was evaporated. The resulting oil was taken up in chloroform (20 ml) and washed with a saturated solution of sodiumhydrogensulfate followed by a saturated solution of sodiumbicarbonate, water and brine. The organic layer was separated, dried and evaporated. The resulting product was checked for purity by TLC (eluent: $CHCl_3$/MeOH: 9/1)
Yield: 1.64 g, solid

Cleavage:

640 mg of the resulting solid Boc-protected compound was dissolved in 3.1 ml ice cold HCl in dioxane (12.98 M, 20 equivalents) and left on ice. The progress of the reaction was monitored by TLC (eluent: $CHCl_3$/MeOH: 9/1). After completion of the reaction the solvent was removed and the resulting oil was taken up in methanole and

evaporated again. After that the resulting oil was dried over phosphorous-V-oxide and triturated two times with diethylether. The purity was checked by HPLC.
Yield: 0.265 g
The purity was checked by HPLC. The identity of the reaction product was checked by NMR analysis.

1.4 Synthesis of glutaminyl pyrrolidine hydrochloride

Acylation:

N-t-Butyl-oxycarbonylglutamine (3.0 g, 12.18 mmol) was dissolved in 7ml THF and brought to –15°C. Into that mixture CAIBE (isobutylchloroformiate) (1,6 ml, 12.18 mmol) and 4-methylmorpholine (1.3 ml, 12.18 mmol) where added and the solution was stirred for 15 min. The formation of the mixed anhydride was checked by TLC (eluent: $CHCl_3$/MeOH: 9/1). After warming to –10°C 1 equivalent of pyrrolidine (1.0 ml, 12.18 mmol) was added. The mixture was brought to room temperature and stirred overnight.

Workup:

The sediment formed was filtered off and the solvent was evaporated. The resulting oil was taken up in chloroform (20 ml) and washed with a saturated solution of sodiumhydrogensulfate followed by a saturated solution of sodiumbicarbonate, water and brine. The organic layer was separated, dried and evaporated. The resulting product was checked for purity by TLC (eluent: $CHCl_3$/MeOH: 9/1)
Yield: 2.7 g solid

Cleavage:

2.7g of the resulting solid was dissolved in 13.0 ml ice cold HCl in dioxane (12.98 M, 20 equivalents) and left on ice. The progress of the reaction was monitored by TLC (eluent: $CHCl_3$/MeOH: 9/1). After completion of the reaction the solvent was removed and the resulting oil was taken up in methanole and evaporated again. After

that the resulting oil was dried over phosphorous-V-oxide and triturated two times with diethylether.

Yield: 980 mg

The purity was checked by HPLC. The identity of the reaction product was checked by NMR analysis.

Example 2: Chemical characterization of selected dipeptide compounds

2.1 Melting point determination

Melting points were determined on a Kofler heating platform microscope from Leica Aktiengesellschaft, the values are not corrected, or on a DSC apparatus (Heumann-Pharma).

2.2 Optical rotation

The rotation values were recorded at different wavelengths on a "Polarimeter 341" or higher, from the Perkin-Elmer company.

2.3 Measurement conditions for the mass spectroscopy

The mass spectra were recorded by means of electrospray ionisation (ESI) on an "API 165" or API 365" from the PE Sciex company. The operation is carried out using an approximate concentration of c = 10 µg/ml, the substance is taken up in MeOH/H_2O 50:50, 0.1 % HCO_2H, the infusion is effected using a spray pump (20 µl/min). The measurement were made in positive mode $[M+H]^+$, the ESI voltage is U=5600V.

2.4. Results

2.4.1 Tests on Isoleucyl thiazolidine fumarate (isomer)

Substance	Mp (°C)	CE (min)	MS	$[\alpha]H_2O$
L-threo-IT*F	150^{DSC}	160	203	-10.7 (405 nm)
D-threo-IT*F	147	158	203	not determined

L-allo-IT*F	145-6	154	203	-4.58 (380 nm)
D-allo-IT*F	144-6	150	203	4.5 (380 nm)

IT*F = Isoleucyl thiazolidine fumarate
The NMR and HPLC data confirm the identity of the substance in question.

2.4.2 Tests on other isoleucyl thiazolidine salts

IT*salt	M ($gmol^{-1}$)	MP (°C)
succinate	522.73	116
tartrate	352.41	122
fumarate	520.71	156
hydrochloride	238.77	169
phosphate	300.32	105

Example 3: Synthesis of Xaa-Pro-Yaa tripeptides

All syntheses were carried out on a peptide synthesizer SP 650 (Labortec AG) applying Fmoc/tBu-strategy. Protected amino acids were purchased from Novabiochem or Bachem. trifluoro acetic acid (TFA) was purchased from Merck, triisopropyl silane (TIS) was purchased from Fluka.

Pre-loaded Fmoc-Yaa-Wang resin (2.8 g/ substitution level 0.57 mmol/g) was deprotected using 20% piperidine/ N,N-dimethylformamide (DMF). After washing with DMF a solution of 2 eq (1.1 g) of Fmoc-Pro-OH were solved in DMF (12ml solvent per gram resin). 2eq (1.04 g) of 2-(1 H-Benzotriazole 1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) and 4 eq (1.11ml) of N,N-diisopropylethylamine (DIEA) were added and placed in the reaction vessel. The mixture was shaken at room temperature for 20 minutes. Then the coupling cycle was repeated. After subsequent washing with DMF, dichlormethane, isopropanol and diethyl ether the resulting Fmoc-Pro-Ile-Wang resin was dried and then divided into 6 parts before coupling the last amino acid derivative.

Fmoc protecting group was removed as described above. After that 0.54 mmol of the Boc-amino acid, 0.54 mmol TBTU and 0.108 mmol DIEA in DMF were shaken for 20 min. The coupling cycle was repeated. Finally the peptide resin was washed and dried described above.

The peptide was cleaved from the resin using a mixture of trifluoroacetic acid (TFA) for 2.5 h, containing the following scavengers: TFA/H_2O/trilsipropylsilane (TIS) = 9.5/0.25/0.25

The yields of crude peptides were 80-90% on the average. The crude peptide was purified by HPLC on a Nucleosil C18 column (7 µm, 250*21.20 mm, 100 A) using a linear gradient of 0.1% TFA/H_2O with increasing concentration of 0.1% TFA/acetonitrile (from 5% to 65% in 40 min) at 6 ml/min.

The pure peptide was obtained by lyophilization, identified by Electrospray mass spectrometry and HPLC analysis.

3.1 Results - Identification of Xaa-Pro-Yaa tripeptides after chemical synthesis

Peptide	Mass (calc.)	Mass (exp.)[1] [M+H$^+$]	HPLC k'[2]
Abu-Pro-Ile	313.4	314.0	5.7
Cha-Pro-Ile	381.52	382.0	10.4
Nva-Pro-Ile	327.43	328.2	6.82
Phg-Pro-Ile	361.44	362.2	7.9
Nle-Pro-Ile	341.45	342.2	8.09
Pip-Pro-Ile	338.56	340.0	6.5
Thr-Pro-Ile	329.4	330.0	5.12
Trp-Pro-Ile	414.51	415.2	9.85
Phe-Pro-Ile	375.47	376.2	8.96
Ser-Pro-Ile	315.37	316.3	5.24
Ser(P)-Pro-Ile	395.37	396.0	3.35
Tyr(P)-Pro-Ile	471.47	472.3	5.14
Val-Pro-Val	313.4	314.0	5.07
Ile-Pro-Val	327.43	328.5	6.41
Ile-Pro-*allo*-Ile	341.4	342.0	7.72
Val-Pro-*allo*-Ile	327.4	328.5	6.51
Tyr-Pro-*allo*-Ile	391.5	392.0	7.02
2-Amino octanoic acid-Pro-Ile	369.5	370.2	10.63
Ser(Bzl)-Pro-Ile	405.49	406.0	9.87
Orn-Pro-Ile	342.42	343.1	3.73
Tic-Pro-Ile	387.46	388.0	8.57
Aze-Pro-Ile	311.4	312.4	5.29
Aib-Pro-Ile	313.4	314.0	5.25
t-butyl-Gly-Pro-Ile	341.47	342.1	7.16
Ile-Hyp-Ile	356.45	358.2	6.57
t-butyl-Gly-Pro-Val	327.4	328.4	6.32
t-butyl-Gly-Pro-Gly	285.4	286.3	3.74
t-butyl-Gly-Pro-Ile-amide	340.47	341.3	7.8
t-butyl Gly-Pro-D-Val	327.4	328.6	7.27
t-butyl-Gly-Pro-*t*-butyl-Gly	341.24	342.5	9.09
Ile-Pro-*t*-butyl-Gly	341,47	342,36	6.93
Val-Pro-*t*-butyl-Gly	327.4	328.15	5.98

[1] [M+H$^+$] were determined by Electrospray mass spectrometry in positive ionization mode.

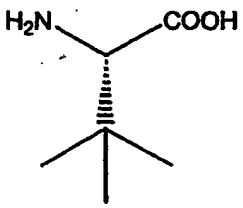
[2] RP-HPLC conditions:

column:	LiChrospher 100 RP 18 (5µm), 125 x 4 mm
detection (UV):	214nm
gradient system:	acetonitrile (ACN)/H_2O (0.1% TFA)
	from 5% ACN to 50% in15 min,
flow :	1 ml/min

$k' = (t_r - t_0)/t_0$

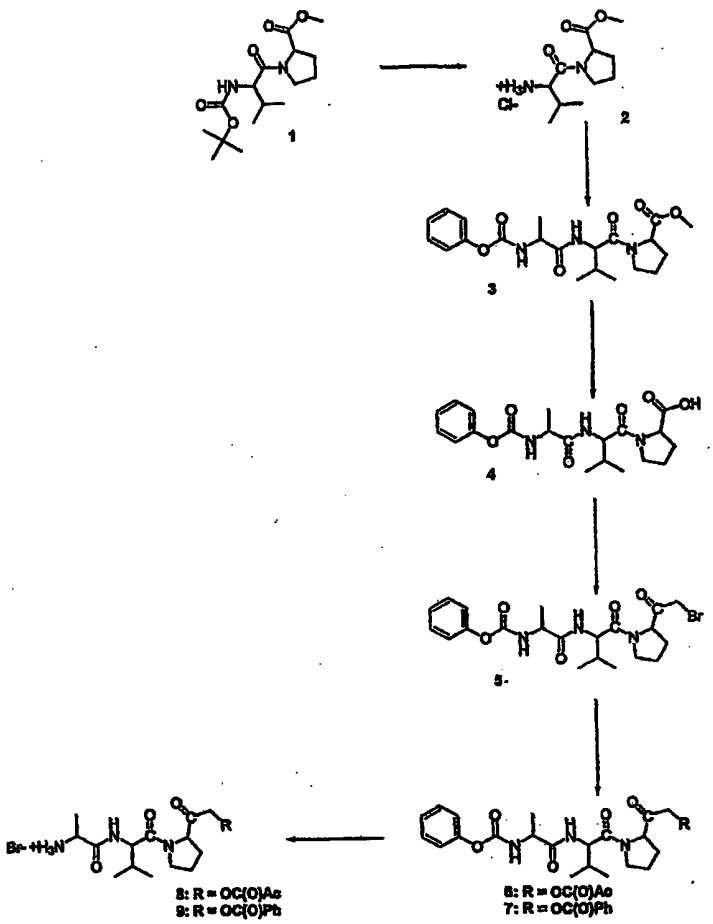
$t_0 = 1.16$ min

t-butyl-Gly is defined as:



Ser(Bzl) and Ser(P) are defined as benzylserine and phosphorylserine, respectively. Tyr(P) is defined as phosphoryltyrosine.

Example 4: Synthesis of peptidylketones



H-Val-Pro-OMe*HCl ***2***

Boc-Val-OH (3.00g, 13.8mmol) was dissolved in 10ml of dry THF and cooled down to –15°C. To the mixture CAIBE (1.80ml, 13.8mmol) and NMM (1.52ml, 13.8mmol) where added and the solution was stirred until the formation of the mixed anhydride was complete. Then the mixture was brought to –10°C and NMM (1.52ml, 13.8mmol) was added followed by H-Pro-OMe*HCl (2.29g, 13.8mmol). The mixture was allowed to reach room temperature and left overnight. After removing the solvent and the usual workup the resulting ester ***1*** was taken without further characterisation. The ester ***1*** was dissolved in HCl/HOAc (5ml, 6N) and left at 0°C until the removal of

the Boc-group was complete. The solvent was then removed and the resulting oil was treated with diethylether to give a white solid *2*.

Yield: 2.5g, 80%

Z-Ala-Val-Pro-OMe *3*

Z-Ala OH (3.5g, 15.7mmol) and *2* (4.18g, 15.7mmol) where treated in the same manner as above for *1*, to give *3* as a white solid.

Yield: 4.2g, 64%

Z-Ala-Val-Pro-OH *4*

3 (4.2g, 9.6mmol) was dissolved in 30 ml of water/acetone (1/5 v/v) and 11.6ml NaOH (1N) where added. After completion of the reaction the organic solvent was removed by evaporation and the resulting solution was diluted by 15ml $NaHCO_3$ solution (saturated). Then the mixture was extracted three times by 10ml of acetic acid ethyl ester. After that the solution was brought to pH2 by adding HCl (15% in water). The resulting mixture was extracted three times by 30ml of acetic acid ethyl ester. The organic layer was separated and washed three times with brine, dried (Na_2SO_4) and evaporated.

Yield: 3.5g ,87%

Z-Ala-Val-Pro-CH_2-Br *5*

4 (2.00g, 4.76mmol) was dissolved in 15ml of dry THF and converted into a mixed anhydride (see compound *1*) using CAIBE (0.623ml, 4.76mmol) and NMM (0.525 ml, 4.76mmol). The precipitate formed was filtered off and cooled down to –15°C. Then diazomethane (23.8mmol in 30ml ether) was dropped into the solution under an argon atmosphere. After leaving the mixture for 1h at 0°C 1.27ml of HBr (33% in AcOH) was added and the solution was stirred for 30min at room temperature. After that 70 ml of ether was added and the mixture was washed with 20 ml of water. The organic layer was separated and dried (Na_2SO_4) and evaporated.

Yield (crude): 1.8g , 80%

Z-protected acyloxymethylene ketones

The acid (2eq) was dissolved in DMF and an equimolar amount of KF was added. The suspension was allowed to stir at room temperature for 1 hour. Then the brommethylene (1eq) component was added and the solution was allowed to stir overnight. After that the solvent was removed under vacuum and the resulting oil was dissolved in chloroform and washed with brine. Then the organic layer was separated dried (Na_2SO_4) and the solvent was removed. The product was purified by column chromatography using silica gel and heptane/chloroform.

Z-Ala-Val-Pro-CH_2O-C(O)-CH_3 ***6***

Acetic acid (230µl, 4.02mmol), KF (0.234g, 4.02mmol), ***5*** (1.00g, 2.01mmol)
Yield: 0.351g, 36%
Z-Ala-Val-Pro-CH_2O-C(O)-Ph ***7***

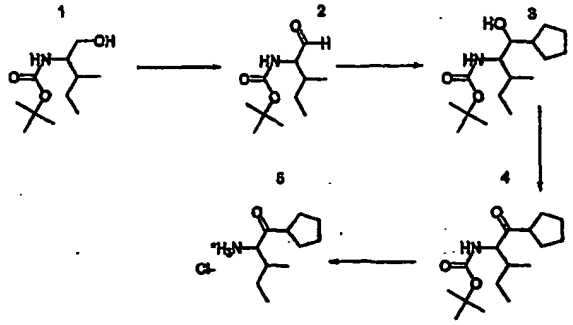
Benzoic acid (0.275g, 2.25mmol), KF (0.131mg, 2.25mmol), ***5*** (0.56g. 1.13mmol)
Yield: 0.34g, 56%
Deprotection

The Z-protected compound was dissolved in HBr/AcOH and stirred. When the reaction was complete ether was added, the white precipitate formed was filtered off and dried.
H-Ala-Val-Pro-CH_2O-C(O)CH_3*HBr ***8***

6 (0.351g, 0,73mmol)
Yield: 0.252g, 98%
H-Ala-Val-Pro-CH_2O-C(O)Ph*HBr ***9***

7 (0.34g, 0.63mmol)
Yield: 0,251g, 99%

Example 5: Synthesis of cycloalkylketones



Boc-isoleucinal ***2***

Oxalylchloride (714 µl, 8.28 mmol) was dissolved in 10 ml of dry dichlormethane and brought to –78°C. Then DMSO (817 µl, 8.28 mmol) was added dropwise. The solution was stirred for 20 min at –78°C. Then ***1*** (1.00 g, 4.6 mmol) was added and the mixture was stirred for 20 min. After that TEA (2.58 ml, 18.4 mmol) was added and the mixture was allowed to reach room temperature. The mixture was diluted with hexane/ethylacetate (2/1 v/v) and 10 ml of HCl (10% in water) was added. The organic layer was separated and the aqueous phase was extracted with 20 ml of methylenechloride. All organic layers were collected and washed with brine, followed by water, then dried. The product was purified by column chromatography using silica gel and heptane/chloroform.

Yield: 0.52 g, 52%

tert-butyl *N*-1-[cyclopentyl(hydroxy)methyl]-2-methylbutylcarbamate ***3***

2 (0.52 g, 2.42 mmol) was dissolved in 10 ml of dry THF and cooled down to 0°C. Then cyclopentylmagnesiumbromide (1.45 ml of a 2 M solution) was added. After completion of the reaction (2 ml) of water was added and solution was neutralized by adding aqueous HCl. Then methylenechloride was added and the organic layer was separated and dried (Na_2SO_4). After evaporation the resulting oil was used without further characterisation.

tert-butyl *N*-[1-(cyclopentylcarbonyl)-2-methylbutyl]carbamate ***4***

3 (0.61 g, 2.15 mmol) was treated like *1*. Oxalylchloride (333 µl, 3.87 mmol), DMSO (382 µl, 5.37 mmol), TEA (1.2 ml, 8.59 mmol)
Yield: 0.180 g, 30%
1-cyclopentyl-3-methyl-1-oxo-2-pentanaminium chloride *5*

4 (0.18 g, 0.63 mmol) was dissolved in 2 ml HCl (7 N in dioxane). After completion of the reaction the solvent was removed and the resulting oil was purified by column chromatography on silical gel using a chloroform/methanol/water gradient. The resulting oil was triturated with ether.

Yield: 0.060 g, 54%

Example 6: Synthesis of side chain modified DPIV-inhibitors

6.1 Synthesis of Boc-glutamyl –thiazolidine (Boc-Glu-Thia)
Reaction of Boc-Glu(OMe)-OH with Thia*HCl according to Method B (see section 6.4 for methods), hydrolysis of Boc-Glu(OMe)-Thia according to Method G

6.1.1 Analytical data for Boc-Glu-Thia

Compound	Empirical formula M_r Synthesis method Yield	MS $[M+H]^+$ TLC: R_f/system m.p.	$[\alpha]^{20}D$ Concentration Solvent	Elemental analysis (calc./ found) %	HPLC R_t [min]/system
Boc-Glu-Thia	$C_{13}H_{22}N_2O_5S$ 318.38 B+G 62 %	319.5 0.52 / A[1] 0.42 / B[1] 115-118°C	-3.1 c = 1 methanol	C:49.04/48.89 H:6.96/6.82 N:8.80/8.59	13.93 / A[2]

[1] Thin-layer chromatography
System A: chloroform/methanol 90:10
System B: benzene/acetone/acetic acid 25:10:0.5
System C: n-butanol/EA/acetic acid/H_2O 1:1:1:1

[2] HPLC separation conditions
Column: Nucleosil C-18, 7µ, 250 mm x 21 mm
Eluant: Isocratic, 40 % ACN/water/0.1 % TFA
Flow rate: 6 ml/min
λ = 220 nm

6.2 Side chain-modified Boc-glutamyl thiazolidines

Boc-Glu-Thia was modified at the γ-carboxylic acid function by introducing radicals of varying size. The radicals were coupled by way of their amino group by forming an amide bond to the γ-carboxylic acid function, with a variety of coupling methods being used depending on the radical. The following amino components were attached to Boc-Glu-Thia using the method stated:

Amino component	Coupling methods (see section 3.4)	Yields
Polyethylene glycol amine (M_r ≈ 8000)	C	93 %
H-Gly-Gly-Gly-OH	D + E	49 %
H-Gly-Gly-Gly-Gly-Gly-OH	D + E	86 %

In 2 cases, purification of the reaction products differs from the general description of synthesis.

Boc-Glu(Gly$_5$)-Thia

The product already precipitates out from the mixture on stirring overnight; it is subsequently filtered off and washed with 0.1N HCl and copious amounts of water and then dried over P_4O_{10} *in vacuo*.

Boc-Glu(PEG)-Thia

In contrast to the general procedure, the starting materials for the synthesis are dissolved in a 500-fold excess of DMF. After the reaction is complete, the DMF is completely removed *in vacuo* and the residue is dissolved in a large amount of methanol. After ether is poured on, to form an upper layer, the product precipitates out together with the unreacted PEG. Fine purification was carried out by preparative

HPLC separation on a gel filtration column (Pharmazia, Sephadex G-25, 90 µm, 260 mm – 100 mm).

Separating conditions: eluant: water; flow rate: 5 ml/min; λ = 220 nm

6.2.2 Synthesis data for side chain-modified Boc-glutamyl thiazolidines

Compound	Empirical formula M_r Yield	MS $[M+H]^+$ TLC/R_f/ system m.p.	$[\alpha]^{20}D$ Concentration Solvent	Elemental analysis (calc./ found) %	HPLC R_t [min]/system
Boc-Glu(Gly_3)-Thia	$C_{19}H_{31}N_5O_8S$ 489.54 49 %	490.5		C:46.62 H:6.38 N:14.31	
Boc-Glu(Gly_5)-Thia	$C_{23}H_{37}N_7O_{10}S$ 603.64 86 %	604.5 0.09 / C decomp. from 202°C	n.dm.	C:45.76/45.60 H:6.18/6.11 N:16.24/16.56	11.93 / A[2]
Boc-Glu(PEG)-Thia	93 %	≈ 8000 (mass emphasis) 52-53°C	n.dm.	n.dm.	n.dm.

[2] HPLC separation conditions
Column: Nucleosil C-18, 7µ, 250 mm x 21 mm
Eluant: isocratic, 40 % ACN/water/0.1 % TFA
Flow rate: 6 ml/min
λ = 220 nm

6.3 Side chain-modified glutamyl thiazolidines

The N-terminal Boc protecting groups were cleaved off the compounds described in Table 6.2.2 using method F. The substances modified with Gly derivatives were purified by preparative HPLC separation and are present as trifluoroacetates. The H-Glu(PEG)-Thia was purified on a gel filtration column in the same manner as the Boc-protected precursor.

6.3.1 Synthesis data for side chain-modified glutamyl thiazolidines

Compound	Empirical formula M_r Yield	MS $[M+H]^+$ TLC/R_f/ system m.p.	$[\alpha]^{20}D$ Concentration Solvent	Elemental analysis (calc./ found) %	HPLC R_t [min]/ system
H-Glu(Gly$_3$)-Thia *TFA	$C_{16}H_{24}N_5O_8SF_3$ 503.45 94 %	503.45 0.32 / C 91-94°C	+4.1 c = 1 methanol	C:38.17/37.56 H:4.80/4.78 N:13.91/13.43	7.84 / C[3]
H-Glu(Gly$_5$)-Thia *TFA	$C_{20}H_{30}N_7O_{10}SF_3$ 617.55 98 %	617.55 0.25 / C 105-107°C	n.dm.	C:38.90/38.82 H:4.90/4.79 N:15.88/15.39	8.22 / C[3]
H-Glu(PEG)-Thia *HCl	92 %	≈ 8000 (mass emphasis)	n.dm.	n.dm.	n.dm.

[3] HPLC separation conditions
Column: Nucleosil C-18, 7µ, 250 mm x 21 mm
Eluant: ACN/water/0.1 % TFA
Gradient: 20 % ACN → 90 % ACN over 30 min
Flow rate: 6 ml/min
λ = 220 nm

n.dm. – not determined *or* not determinable

6.4 General synthesis procedures

Method A: Peptide bond attachment by the mixed anhydride method using CFIBE as activation reagent

10 mmol of N-terminally protected amino acid or peptide are dissolved in 20 ml of absolute THF. The solution is cooled to -15°C ± 2°C. With stirring in each case, 10 mmol of N-MM and 10 mmol of chloroformic acid isobutyl ester are added in succession, the stated temperature range being strictly adhered to. After approximately 6 min, 10 mmol of the amino component is added. When the amino component is a salt, a further 10 mmol of N-MM is then added to the reaction mixture. The reaction mixture is then stirred for 2 h in the cold state and overnight at room temperature.

The reaction mixture is concentrated using a rotary evaporator, taken up in EA, washed with 5 % KH_2SO_4 solution, saturated $NaHCO_3$ solution and saturated NaCl solution and dried over $NaSO_4$. After removal of the solvent *in vacuo*, the compound is recrystallized from EA/pentane.

Method B: Peptide bond attachment by the mixed anhydride method using pivalic acid chloride as activation reagent

10 mmol of N-terminally protected amino acid or peptide are dissolved in 20 ml of absolute THF. The solution is cooled to 0°C. With stirring in each case, 10 mmol of N-MM and 10 mmol of pivalic acid chloride are added in succession, the stated temperature range being strictly adhered to. After approximately 6 min, the mixture is cooled to -15°C and, once the lower temperature has been reached, 10 mmol of the amino component is added. When the amino component is a salt, a further 10 mmol of N-MM is then added to the reaction mixture. The reaction mixture is then stirred for 2 h in the cold state and overnight at room temperature.

Further working up is carried out as in *Method A*.

Method C: Peptide bond attachment using TBTU as activation reagent

10 mmol of the N-terminally protected amino acid or peptide and 10 mmol of the C-terminally protected amino component are dissolved in 20 ml of absolute DMF. The solution is cooled to 0°C. With stirring in each case, 10 mmol of DIPEA and 10 mmol of TBTU are added in succession. The reaction mixture is stirred for one hour at 0°C and then overnight at room temperature. The DMF is completely removed *in vacuo* and the product is worked up as described in *Method A*.

Method D: Synthesis of an active ester (N-hydroxysuccinimide ester)

10 mmol of N-terminally protected amino acid or peptide and 10 mmol of N-hydroxysuccinimide are dissolved in 20 ml of absolute THF. The solution is cooled to

0°C and 10 mmol of dicyclohexylcarbodiimide are added, with stirring. The reaction mixture is stirred for a further 2 h at 0°C and then overnight at room temperature. The resulting N,N'-dicyclohexylurea is filtered off and the solvent is removed *in vacuo* and the remaining product is recrystallized from EA/pentane.

Method E: Amide bond attachment using N-hydroxysuccinimide esters

10 mmol of the C-terminally unprotected amino component is introduced into an $NaHCO_3$ solution (20 mmol in 20 ml of water). At room temperature and with stirring, 10 mmol of the N-terminally protected N-hydroxysuccinimide ester dissolved in 10 ml of dioxane are slowly added dropwise. Stirring of the reaction mixture is continued overnight and the solvent is then removed *in vacuo*.

Further working up is carried out as in *Method A*.

Method F: Cleavage of the Boc protecting group

3 ml of 1.1N HCl/glacial acetic acid (*Method F1*) or 3 ml of 1.1N HCl/dioxane (*Method F2*) or 3 ml of 50 % TFA in DCM (*Method F3*) are added to 1 mmol of Boc-protected amino acid pyrrolidide, thiazolidide or peptide. The cleavage at RT is monitored by means of TLC. After the reaction is complete (approximately 2 h), the compound is precipitated in the form of the hydrochloride using absolute diethyl ether and is isolated with suction and dried over P_4O_{10} *in vacuo*. Using methanol/ether, the product is recrystallized or reprecipitated.

Method G: Hydrolysis

1 mmol of peptide methyl ester is dissolved in 10 ml of acetone and 11 ml of 0.1M NaOH solution and stirred at room temperature. The course of the hydrolysis is monitored by means of TLC. After the reaction is complete, the acetone is removed *in vacuo*. The remaining aqueous solution is acidified, using concentrated KH_2SO_4 solution, until a pH of 2-3 is reached. The product is then extracted several times

using EA; the combined ethyl acetate fractions are washed with saturated NaCl solution and dried over $NaSO_4$, and the solvent is removed *in vacuo*. Crystallization from EA/pentane is carried out.

Example 7: K_i-determination

For K_i determination, dipeptidyl peptidase IV from porcine kidney with a specific activity against glycylprolyl-4-nitroaniline of 37.5 U/mg and an enzyme concentration of 1.41 mg/ml in the stock solution was used.

Assay mixture:

100 µl test compound in a concentration range of $1*10^{-5}$ M – $1*10^{-8}$ M respectively were admixed with 50 µl glycylprolyl-4-nitroaniline in different concentrations (0.4 mM, 0.2 mM, 0.1 mM, 0,05 mM) and 100 µl HEPES (40 mM, pH7.6; ion strength = 0.125). The assay mixture was pre-incubated at 30 °C for 30 min. After pre-incubation, 20 µl DPIV (1:600 diluted) was added and measurement of yellow color development due to 4-nitroaniline release was performed at 30°C and λ = 405 nm for 10 min. using a plate reader (HTS7000 plus, Applied Biosystems, Weiterstadt, Germany).

The K_i-values were calculated using Graphit version 4.0.13, 4.0.13 and 4.0.15 (Erithacus Software, Ltd, UK).

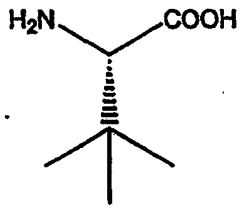
7.1 Results - KI values of DPIV Inhibition

Compound	KI [M]
H-Asn-pyrrolidine	$1.20*10^{-5}$
H-Asn-thiazolidine	$3.5*10^{-6}$
H-Asp-pyrrolidine	$1.4*10^{-8}$
H-Asp-thiazolidine	$2.9*10^{-6}$
H-Asp(NHOH)-pyrrolidine	$1.3*10^{-5}$
H-Asp(NHOH)-thiazolidine	$8.8*10^{-6}$

H-Glu-pyrrolidine	$2.2*10^{-6}$
H-Glu-thiazolidine	$6.1*10^{-7}$
H-Glu(NHOH)-pyrrolidine	$2.8*10^{-6}$
H-Glu(NHOH)-thiazolidine	$1.7*10^{-6}$
H-His-pyrrolidine	$3.5*10^{-6}$
H-His-thiazolidine	$1.8*10^{-6}$
H-Pro-pyrrolidine	$4.1*10^{-6}$
H-Pro-thiazolidine	$1.2*10^{-6}$
H-Ile-azididine	$3.1*10^{-6}$
H-Ile-pyrrolidine	$2.1*10^{-7}$
H-L-*threo*-Ile-thiazolidine	$8.0*10^{-8}$
H-L-*allo*-Ile-thiazolidine	$1.9*10^{-7}$
D-*threo*-isoleucyl-thiazolidine-fumarate	no inhibition
D-*allo*-isoleucyl-thiazolidine-fumarate	no inhibition
H-L-*threo*-Ile-thiazolidine-succinate	$5.1*10^{-8}$
H-L-*threo*-Ile-thiazolidine-tartrate	$8.3*10^{-8}$
H-L-*threo*-Ile-thiazolidine-fumarate	$8.3*10^{-8}$
H-L-*threo*-Ile-thiazolidine-hydrochloride	$7.2*10^{-8}$
H-L-*threo*-Ile-thiazolidine-phosphate	$1.3*10^{-7}$
H-Val-pyrrolidine	$4.8*10^{-7}$
H-Val-thiazolidine	$2.7*10^{-7}$
Diprotin A	$3.45*10^{-6}$
Diprotin B	$2.24*10^{-5}$
Nva-Pro-Ile	$6.17*10^{-6}$
Cha-Pro-Ile	$5.99*10^{-6}$
Nle-Pro-Ile	$9.60*10^{-6}$
Phe-Pro-Ile	$1.47*10^{-5}$
Val-Pro-Val	$4.45*10^{-6}$
Ile-Pro-Val	$5.25*10^{-6}$
Abu-Pro-Ile	$8.75*10^{-6}$
Ile-Pro-*allo*-Ile	$5.22*10^{-6}$
Val-Pro-*allo*-Ile	$9.54*10^{-6}$
Tyr-Pro-*allo*-Ile	$1.82*10^{-5}$
AOA-Pro-Ile	$1.26*10^{-5}$
t-butyl-Gly-Pro-Ile	$3.10*10^{-6}$
Ser(Bzl)-Pro-Ile	$2.16*10^{-5}$
Aze-Pro-Ile	$2.05*10^{-5}$
t-butyl-Gly-Pro-Val	$3.08*10^{-6}$
Gln-Pyrr	$2.26*10^{-6}$

Gln-Thia	$1.21*10^{-8}$
Val-Pro-*t*-butyl-Gly	$1.96*10^{-5}$
t-butyl-Gly-Pro-Gly	$1.51*10^{-5}$
Ile-Pro-*t*-butyl-Gly	$1.89*10^{-5}$
t-butyl-Gly-Pro-IleNH$_2$	$5.60*10^{-6}$
t-butyl-Gly-Pro-D-Val	$2.65*10^{-5}$
t-butyl-Gly-Pro-*t*-butyl-Gly	$1.41*10^{-5}$
Ile-cyclopentyl ketone	$6.29*10^{-6}$
t-butyl-Gly-cyclohexyl ketone	$2.73*10^{-4}$
Ile-cyclohexyl ketone	$5.68*10^{-5}$
Val-cyclopentyl ketone	$1.31*10^{-5}$
Val-Pro- methyl ketone	$4.76*10^{-8}$
Val-Pro- acyloxy methyl ketone	$1.05*10^{-9}$
Val-Pro- benzoyl methyl ketone	$5.36*10^{-10}$
Val-Pro-benzothiazol methyl ketone	$3.73*10^{-8}$
H-Glu-Thia	$6.2*10^{-7}$
H-Gly(NHOH)-Thia	$1.7*10^{-6}$
H-Glu(Gly$_3$)-Thia	$1.92*10^{-8}$
H-Glu(Gly$_5$)-Thia	$9.93*10^{-8}$
H-Glu(PEG)-Thia	$3.11*10^{-6}$

t-butyl-Gly is defined as:



Ser(Bzl) and Ser(P) are defined as benzyl-serine and phosphoryl-serine, respectively. Tyr(P) is defined as phosphoryl-tyrosine.

Example 8: Determination of IC_{50}-Values

100 µl inhibitor stock solution were mixed with 100 µl buffer (HEPES pH7.6) and 50 µl substrate (Gly-Pro-pNA, final concentration 0.4 mM) and preincubated at 30°C. Reaction was started by addition of 20 µl purified porcine DPIV. Formation of the product pNA was measured at 405 nm over 10 min using the HTS 7000Plus plate

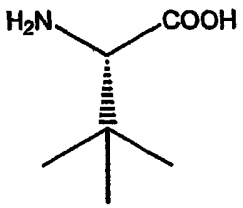
reader (Perkin Elmer) and slopes were calculated. The final inhibitor concentrations ranged between 1 mM and 30 nM. For calculation of IC50 GraFit 4.0.13 (Erithacus Software) was used.

8.1 Results – Determination of IC_{50} values

Compound	IC50 [M]
Isoleucyl thiazolidine fumarate	$1.28*10^{-7}$
Diprotin A	$4.69*10^{-6}$
Diprotin B	$5.54*10^{-5}$
Phg-Pro-Ile	$1.54*10^{-4}$
Nva-Pro-Ile	$2.49*10^{-5}$
Cha-Pro-Ile	$2.03*10^{-5}$
Nle-Pro-Ile	$2.19*10^{-5}$
Ser(P)-Pro-Ile	0.012
Tyr(P)-Pro-Ile	0.002
Phe-Pro-Ile	$6.20*10^{-5}$
Trp-Pro-Ile	$3.17*10^{-4}$
Ser-Pro-Ile	$2.81*10^{-4}$
Thr-Pro-Ile	$1.00*10^{-4}$
Val-Pro-Val	$1.64*10^{-5}$
Ile-Pro-Val	$1.52*10^{-5}$
Abu-Pro-Ile	$3.43*10^{-5}$
Pip-Pro-Ile	0.100
Ile-Pro-*allo*-Ile	$1.54*10^{-5}$
Val-Pro-*allo*-Ile	$1.80*10^{-5}$
Tyr-Pro-*allo*-Ile	$6.41*10^{-5}$
AOA-Pro-Ile	$4.21*10^{-5}$
t-butyl-Gly-Pro-Ile	$9.34*10^{-6}$
Ser(Bzl)-Pro-Ile	$6.78*10^{-5}$
Tic-Pro-Ile	0.001
Orn-Pro-Ile	$2.16*10^{-4}$
Gln-Thia	$5.27*10^{-6}$
Aze-Pro-Ile	$7.28*10^{-5}$
Ile-Hyp-Ile	0.006
t-butyl-Gly-Pro-Val	$1.38*10^{-5}$
Gln-Pyrr	$1.50*10^{-5}$
Val-Pro-*t*-butyl-Gly	$6.75*10^{-5}$

t-butyl-Gly-Pro-Gly	$5.63*10^{-5}$
Ile-Pro-*t*-butyl-Gly	$8.23*10^{-5}$
t-butyl-Gly-Pro-IleNH$_2$	$2.29*10^{-5}$
t-butyl-Gly-Pro-D-Val	$1.12*10^{-4}$
t-butyl-Gly-Pro-*t*-butyl-Gly	$2.45*10^{-5}$
Aib-Pro-Ile	no inhibition
Ile-cyclopentyl ketone	$3.82*10^{-5}$
t-butyl-Gly-cyclohexyl ketone	$2.73*10^{-4}$
Ile-cyclohexyl ketone	$2.93*10^{-4}$
Val-cyclopentyl ketone	$4.90*10^{-5}$
Val-cyclohexyl ketone	0.001
Val-Pro- methyl ketone	$5.79*10^{-7}$
Val-Pro- acyloxy methyl ketone	$1.02*10^{-8}$
Val-Pro- benzoyl methyl ketone	$1.79*10^{-8}$
Val-Pro-benzothiazol methyl ketone	$1.38*10^{-7}$

t-butyl-Gly is defined as:



Ser(Bzl) and Ser(P) are defined as benzyl-serine and phosphoryl-serine, respectively. Tyr(P) is defined as phosphoryl-tyrosine.

Example 9: Inhibition of DPIV-like enzymes – dipeptidyl peptidase II

DP II (3.4.14.2) releases N-terminal dipeptides from oligopeptides if the N-terminus is not protonated (McDonald, J.K., Ellis, S. & Reilly, T.J., 1966, *J. Biol. Chem.*, 241, 1494-1501). Pro and Ala in P_1-position are preferred residues. The enzyme activity is described as DPIV-like activity, but DP II has an acidic pH-optimum. The enzyme used was purified from porcine kidney.

Assay:

100 µl glutaminyl pyrrolidine or glutaminyl thiazolidine in an concentration range of $1*10^{-4}$ M – $5*10^{-8}$ M were admixed with 100 µl µl buffer solution (40 mM HEPES, pH7.6, 0.015% Brij, 1 mM DTT), 50 µl lysylalanylaminomethylcoumarine solution (5 mM) and 20 µl porcine DP II (250fold diluted in buffer solution). Fluorescence measurement was performed at 30°C and $\lambda_{exdatation}$ = 380 nm, $\lambda_{emission}$ = 465 nm for 25 min using a plate reader (HTS7000plus, Applied Biosystems, Weiterstadt, Germany). The K_i-values were calculated using Graphit 4.0.15 (Erithacus Software, Ltd., UK) and were determined as $K_i = 8.52*10^{-5}$ M ± $6.33*10^{-6}$ M for glutaminyl pyrrolidine and K_i = $1.07*10^{-5}$ M ± $3.81*10^{-7}$ M for glutaminyl thiazolidine.

Example 10: Cross Reacting Enzymes

Glutaminyl pyrrolidine and glutaminyl thiazolidine were tested for their cross reacting potency against dipeptidyl peptidase I, prolyl oligopeptidase and prolidase.

Dipeptidyl peptidase I (DP I, cathepsin C):

DP I or cathepsin C is a lysosomal cysteine protease which cleaves off dipeptides from the N-terminus of their substrates (Gutman, H.R. & Fruton, J.S., 1948, *J. Biol: Chem.*, 174, 851-858) . It is classified as a cysteine protease. The enzyme used was purchased from Qiagen (Qiagen GmbH, Hilden, Germany). In order to get a fully active enzyme, the enzyme was diluted 1000fold in MES buffer pH5,6 (40 mM MES, 4 mM DTT, 4 mM KCl, 2 mM EDTA, 0.015% Brij) and pre-incubated for 30 min at 30°C.

Assay:

50 µl glutaminyl pyrrolidine or glutaminyl thiazolidine in a concentration range of $1*10^{-5}$ M – $1*10^{-7}$ M were admixed with 110 µl buffer-enzyme-mixture. The assay mixture was pre-incubated at 30 °C for 15 min. After pre-incubation, 100 µl histidylseryl- -nitroaniline ($2*10^{-5}$M) was added and measurement of yellow color development due to β-nitroaniline release was performed at 30°C and $\lambda_{excitation}$ = 380

nm, $\lambda_{emission}$ = 465 nm for 10 min., using a plate reader (HTS7000 plus, Applied Biosystems, Welterstadt, Germany).

The IC_{50}-values were calculated using Graphit 4.0.15 (Erithacus Software, Ltd., UK). No inhibition of the DP I enzyme activity by glutaminyl pyrrolidine or glutaminyl thiazolidine was found.

Prolyl oligopeptidase (POP)

Prolyl oligopeptidase (EC 3.4.21.26) is a serine type endoprotease which cleaves off peptides at the N-terminal part of the Xaa-Pro bond (Walter, R., Shlank, H., Glass, J.D., Schwartz,I.L. & Kerenyi, T.D., 1971, *Science*, 173, 827-829). Substrates are peptides with a molecular weight up to 3000 Da. The enzyme used was a recombinant human prolyl oligopeptidase. Recombinant expression was performed in *E. coli* under standard conditions as described elsewhere in the state of the art.

Assay:

100 µl glutaminyl pyrrolidine or glutaminyl thiazolidine in an concentration range of $1*10^{-4}$ M – $5*10^{-8}$ M were admixed with 100 µl µl buffer solution (40 mM HEPES, pH7.6, 0.015% Brij, 1 mM DTT) and 20 µl POP solution. The assay mixture was pre-incubated at 30 °C for 15 min. After pre-incubation, 50 µl glycylprolylprolyl-4-nitroanlline solution (0.29 mM) was added and measurement of yellow color development due to 4-nitroanlline release was performed at 30°C and λ = 405 nm for 10 min using a plate reader (sunrise, Tecan, Crailsheim, Germany). The IC_{50}-values were calculated using Graphit 4.0.15 (Erithacus Software, Ltd., UK). No inhibition of POP activity by glutaminyl pyrrolidine or glutaminyl thiazolidine was found.

Prolidase (X-Pro dipeptidase)

Prolidase (EC 3.4.13.9) was first described by Bergmann & Fruton (Bergmann, M. & Fruton, JS, 1937, *J. Biol. Chem.* 189-202). Prolidase releases the N-terminal amino acid from Xaa-Pro dipeptides and has a pH optimum between 6 and 9.

Prolidase from porcine kidney (ICN Biomedicals, Eschwege, Germany) was solved (1mg/ml) in assay buffer (20mM $NH_4(CH_3COO)_2$, 3mM $MnCl_2$, pH 7.6). In order to get a fully active enzyme the solution was incubated for 60 min at room temperature.

Assay:

450 µl glutaminyl pyrrolidine or glutaminyl thiazolidine in an concentration range of $5*10^{-3}$ M – $5*10^{-7}$ M were admixed with 500 µl buffer solution (20mM $NH_4(CH_3COO)_2$, pH 7.6) and 250 µl Ile-Pro-OH (0.5mM in the assay mixture). The assay mixture was pre-incubated at 30 °C for 5 min. After pre-incubation, 75 µl Prolidase (1:10 diluted in assay buffer) were added and measurement was performed at 30°C and λ = 220 nm for 20 min using a UV/Vis photometer, UV1 (Thermo Spectronic, Cambridge, UK).

The IC 50-values were calculated using Graphit 4.0.15 (Erithacus Software, Ltd., UK). They were determined as IC_{50} > 3mM for glutaminyl thiazolidine and as IC_{50} = $3.4*10^{-4}$M ± $5.63*10^{-5}$ for glutaminyl pyrrolidine.

Example 11: Determination of DPIV inhibiting activity after intravasal and oral administration to Wistar rats

Animals

Male Wistar rats (Shoe: Wist(Sho)) with a body weight ranging between 250 and 350 g were purchased from Tierzucht Schönwalde (Schönwalde, Germany).

Housing conditions

Animals were single-caged under conventional conditions with controlled temperature (22±2 °C) on a 12/12 hours light/dark cycle (light on at 06:00 AM). Standard pelleted chow (ssniff® Soest, Germany) and tap water acidified with HCl were allowed *ad libitum*.

Catheter insertion into carotid artery

After ≥one week of adaptation at the housing conditions, catheters were implanted into the carotid artery of Wistar rats under general anaesthesia (i.p. injection of 0.25 ml/kg b.w. Rompun® [2 %], BayerVital, Germany and 0.5 ml/kg b.w. Ketamin 10, Atarost GmbH & Co., Twistringen, Germany). The animals were allowed to recover for one week. The catheters were flushed with heparin-saline (100 IU/ml) three times per week. In case of catheter dysfunction, a second catheter was inserted into the contra-lateral carotid artery of the respective rat. After one week of recovery from surgery, this animal was reintegrated into the study. In case of dysfunction of the second catheter, the animal was withdrawn from the study. A new animal was recruited and the experiments were continued in the planned sequence, beginning at least 7 days after catheter implantation.

Experimental design

Rats with intact catheter function were administered placebo (1 ml saline, 0.154 mol/l) or test compound via the oral and the intra-vasal (intra-arterial) route.
After overnight fasting, 100 µl samples of heparinised arterial blood were collected at –30, -5, and 0 min. The test substance was dissolved freshly in 1.0 ml saline (0.154 mol/l) and was administered at 0 min either orally via a feeding tube (75 mm; Fine Science Tools, Heidelberg, Germany) or via the intra-vasal route. In the case of oral administration, an additional volume of 1 ml saline was injected into the arterial catheter. In the case of intra-arterial administration, the catheter was immediately flushed with 30 µl saline and an additional 1 ml of saline was given orally via the feeding tube.

After application of placebo or the test substances, arterial blood samples were taken at 2.5, 5, 7.5, 10, 15, 20, 40, 60 and 120 min from the carotid catheter of the conscious unrestrained rats. All blood samples were collected into ice cooled Eppendorf tubes (Eppendorf-Netheler-Hinz, Hamburg, Germany) filled with 10 µl 1M sodium citrate buffer (pH 3.0) for plasma DPIV activity measurement. Eppendorf tubes were centrifuged immediately (12000 rpm for 2 min, Hettich Zentrifuge EBA 12, Tuttlingen; Germany): The plasma fractions were stored on ice until analysis or were frozen at –20 °C until analysis. All plasma samples were labelled with the following data:

- Code number
- Animal Number
- Date of sampling
- Time of sampling

Analytical Methods

The assay mixture for determination of plasma DPIV activity consisted of 80 µl reagent and 20 µl plasma sample. Kinetic measurement of the formation of the yellow product 4-nitroanilline from the substrate glycylprolyl-4-nitroaniline was performed at 390 nm for 1 min at 30 °C after 2 min pre-incubation at the same temperature. The DPIV activity was expressed in mU/ml.

Statistical methods

Statistical evaluations and graphics were performed with PRISM® 3.02 (GraphPad Software, Inc.). All parameters were analysed in a descriptive manner including mean and SD.

11.1 Results – In vivo DPIV-inhibition at t_{max}

STRUCTURE	Dose (mg/kg)	i.v. (%)	p.o. (%)
Gln-Pyrr	100	80	67
Gln-Thia	100	88	71
Diprotin A	100	73	no inhibition
Diprotin B	100	50	no inhibition
Tyr(P)-Pro-Ile	100	37	no inhibition
t-butyl-Gly-Pro-Ile	100	71	28
t-butyl-Gly-Pro-Val	100	72	25
Ala-Val-Pro-acyloxy methyl ketone	100	89	86
Ala-Val-Pro-benzoyl-methyl ketone	100	97	76
Ile-cyclopentyl ketone	100	34	15

Example 12: Action of side chain-modified glutamyl thiazolidines as non-readily-transportable DPIV-inhibitors

Side chain-modified glutamyl thiazolidines having a structure H-Glu(X)-Thia were synthesised, with polyethylene glycol or glycine oligomers of various chain lengths being used as X (see Method A of example for description of synthesis). The binding characteristics of those derivatives and their transportability by the peptide transporter PepT1 were investigated.

Surprisingly, it was found that the side chain modifications alter the binding characteristics of the compounds to DPIV only to a slight extent. In contrast, the ability of the inhibitors to be transported by the peptide transporter is dramatically diminished by the side chain modification.

Side chain modified inhibitors of DPIV or DPIV-like enzymes are therefore well suited to achieving site directed inhibition of DPIV in the body.

12.1 Results: Transportability of selected DPIV-inhibitors.

Compound *amino acid thiazolidines*	**EC50 (mM)** [1]	**I_{max} (nA)** [2]
H-Ile-Thia	0.98	25 ± 8
H-Glu-Thia	1.1	35 ± 13
side chain-modified glutamylthiazolidines		
H-Gly(NHOH)-Thia	3.18	42 ± 11
H-Glu(Gly_3)-Thia	8.54	n.d. [3]
H-Glu(Gly_5)-Thia	> 10	n.d. [3]
H-Glu(PEG)-Thia	> 10	n.d. [3]

[1] Effective concentrations of the compounds inhibiting the binding of ^{3}H-D-Phe-Ala (80mM) to PepT1-expressing *P. pastoris* cells by 50 % (EC_{50} values)

[2] Transport characteristics at PepT1-expressing oocytes of X. leavis – by means of two-electrode voltage clamp method, I = inward currents generated by the transport

Example 13: *In vivo* cancer cell adhesion assay

Using a novel *in vivo* adhesion assay which takes advantage of vital dye labeled tumor cells and their detection in the target tissue *in situ* (von Hörsten et al, 2000), the current example investigates whether the *in vivo* adhesion of MADB106 tumor cells differs in DPIV in treated wild type F344 rats and F344 substrains with a mutation of the DPIV gene.

Animals, injection of tumor cells and processing of lungs

F344USA, F344JAP and F344GER substrains were obtained from a breeding colony at the Central Animal Laboratory at Hannover Medical School, Germany. All substrains were bred for one generation and maintained in a specific-pathogen-free facility at 25°C under a 12h light-12h dark cycle (light on at 07.00 h), with *ad libitum* access to food and water. The exact number of animals used per experiment is indicated by the *F* values with at least four animals per condition and time point.

Cell culture, injection of tumor cells, dissection of the animals and immunohistochemistry were conducted as previously described (von Hörsten et al., 2000). In brief, 1×10^6 MADB106 tumor cells derived from log phase of tumor growth were injected via the lateral tail vein and lungs removed at different time points thereafter. For *in situ* quantification of tumor cells at early time points after injection (30min), cells were vital dye stained using the fluorescein derivate 5-(and -6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) before injection. For quantification of lung surface colonies at later time points (2 weeks after tumor cell inoculation), en-bloc dissected lungs and the heart were injected with 8ml Bouin's solution (72% saturated picric acid solution, 23% formaldehyde, and 5% glacial acetic acid) and fixed in the same solution until lung surface nodules were counted (see below).

Experiments

Three experiments were conducted:

Effect of a single injection of isoleucyl thiazolidine fumarate (2mg i.v. + isoleucyl thiazolidine fumarate 2mg i.p.) on lung tumor colonization in F344USA wild-type rats;

Effect of single injection of isoleucyl cyanopyrrolidine (0.1mg i.v. + 0.1mg i.p.) on tumor cell adhesion to lungs of F344USA wild-type rats;

Effect of single injection of valyl-pyrrolidine fumarate (0.1mg i.v. + 0.1mg i.p.) on tumor cell adhesion to lungs of F344USA wild-type rats;

Immunohistochemistry of CFSE-labeled tumor cells in lungs

Immunostaining of CFSE-labeled MADB106 tumor cells was achieved using mAb characterizing the intracellular CFSE antigen (anti-CFSE; mAb DE1, Boehringer, Mannheim, Germany; mouse, 1:100). For immunohistochemistry, one or two consecutive APAAP stainings were performed as previously described (von Hörsten et al, 2000). Control sections were included in which one or both primary antibodies were omitted.

Quantification of tumor targets: In vivo/in situ cell adhesion assay

Vital dye (Carboxyfluorescein; CFSE) labeling of MADB106 tumor cells allows the quantification of tumor cells and NK cells in thick sections of lung tissue by stereology *in situ* (von Hörsten et al, 2000). In the present study we produced thin sections (8 µm) of the same lungs (n = 10) and performed additional microscopic counting by image analysis of DE1 positive cells. This was done to further simplify the previously validated stereological quantification technique. Therefore, in the present study, the assessment of DE1 positive tumor cells in lung tissue from different substrains 30 min after tumor inoculation was carried out using image analysis approach. All CFSE-labeled MADB106 tumor cells and leukocyte subsets within a grid on the ocular lens were counted (Zeiss Kpl-W 12.5×; grid 0.75 × 0.75 mm = 0.5625

mm^2/grid, using a Zeiss Neofluar objective, ×10, NA = 0.3). Each right upper lobe of the lungs was sectioned at 6 randomly chosen non-adjacent levels. From each level, three sections were evaluated. On average, 30 grid numbers per section were examined (i.e. 0.5625 mm^2/grid × 30 grids × 3 sections × 6 levels) resulting in an area per animal of 3.04 cm^2.

Quantification of macrometastasis on lungs

For quantification of lung surface colonies at later time points (2 weeks after tumor cell inoculation), en-bloc dissected lungs and the heart were injected with 8ml Bouin's solution (72% saturated picric acid solution, 23% formaldehyde, and 5% glacial acetic acid) and fixed in the same solution until lung surface nodules were counted. Three areas per lungs were examined using a gauge (1 cm^2) and lung surface colony numbers were expressed as mean / cm^2 according to the method of Wexler (Wexler, 1966).

Statistical analysis

Data from *in vivo* adhesion assay were analyzed by one-way ANOVAs and Fisher's PLSD post hoc tests, if appropriate. An asterisk indicates significant post hoc effects vs. saline (SHAM) treated controls obtained by Fisher's PLSD. All data are presented as means ± S.E.M.

Results

Effect of a single injection of isoleucyl thiazolidine fumarate on lung tumor colonization in F344USA rats

The number of lung surface tumor colonies after single isoleucyl thiazolidine fumarate administration in F344 rats 2 weeks after injection of MADB106 tumor cells is illustrated in Fig. 1. One factor ANOVA revealed no significant effect ($F(1,12) = 3.2$; $p = 0.1$n.s.) on colony numbers. A trend toward decreased colony numbers in experimental rats was evident.

Effect of single injection of isoleucyl cyanopyrrolidine TFA on tumor adhesion of F344USA rats

The mean number of CFSE positive cells in lung tissue at 30 min after inoculation of MADB106 tumor cells after isoleucyl cyanopyrrolidine TFA treatment is illustrated in Fig. 2. ANOVA showed no significant effect of "treatment" ($F(1,18) = 0.1$; $p = 0.8$n.s.).

Effect of single injection of valyl pyrrolidine fumarate on tumor adhesion of F344USA rats

The mean number of CFSE positive cells in lung tissue at 30 min after inoculation of MADB106 tumor cells after valyl pyrrolidine fumarate treatment is illustrated in Fig. 3. ANOVA showed no significant effect of "treatment" ($F(1,18) = 0.6$; $p = 0.5$n.s.).

Discussion

Tumor cell adhesion and colonization is significantly modified by single injection of isoleucyl thiazolidine fumarate only in mutant F344 substrains suggesting an interaction of the ligand with mutant DPIV and tumor cells. Since the ligand did not significantly affect tumor adhesion in wild type F344USA rats, this may indicate that compound is not interacting with the binding site of MADB106 tumor cells.

Example 14: Cancer Colonization Assays

In the previous example it was demonstrated that MADB106 tumor cell adhesion is significantly modified by a single administration of isoleucyl thiazolidine fumarate only in mutant F344 substrains but not in wild type DPIV expressing F344USA rats. DPIV inhibitors/ligands may interact with the growth of tumor metastases exhibiting properties similar to chemotherapeutic compounds and/or

immunotherapeutical compounds. In contrast to that, thhis example investigates whether the tumor colonization of MADB106 tumor cells differs in chronically DPIV-inhibitor treated wild type F344 rats.

Animals, injection of tumor cells and processing of lungs

F344USA rats were obtained from a breeding colony at the Central Animal Laboratory at Hannover Medical School, Germany. All rats were bred at least for one generation and maintained in a specific-pathogen-free facility at 25°C under a 12h light-12h dark cycle (light on at 07.00 h), with *ad libitum* access to food and water. The exact number of animals used per experiment is indicated by the *F* values with at least four animals per condition and time point.

Cell culture, injection of tumor cells, dissection of the animals and immunohistochemistry were conducted as previously described (von Hörsten et al., 2000). In brief, 1×10^6 MADB106 tumor cells derived from log phase of tumor growth were injected via the lateral tail vein and lungs removed at different time points thereafter. For *in situ* quantification of tumor cells at early time points after injection (30min), cells were vital dye stained using the fluorescein derivate 5-(and -6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) before injection. For quantification of lung surface colonies at later time points (2 weeks after tumor cell inoculation), en-bloc dissected lungs and the heart were injected with 8ml Bouin's solution (72% saturated picric acid solution, 23% formaldehyde, and 5% glacial acetic acid) and fixed in the same solution until lung surface nodules were counted (see below).

Experiments

Two experiments were conducted:

Effect of chronic infusion of different dosages of isoleucyl thiazolidine fumarate (0mg, 0.04mg, 0.4mg, 4mg/24h intragastral via implanted osmotic minipumps) on body weight change and lung tumor colonization in F344USA wild-type rats

Effect of chronic infusion of isoleucyl thiazolidine fumarate (4mg/24h intragastral via implanted osmotic minipumps), lisoleucyl cyanopyrrolidine TFA (0.1mg/24h intragastral via implanted osmotic minipumps) and valyl pyrrolidine fumarate (0.1mg/24h intragastral via implanted osmotic minipumps) on lung tumor colonization in F344USA wild-type rats.

Implantation of osmotic minipumps for chronic intragastric infusion of compounds

Osmotic minipumps (Alzet model 2ML4; flow rate, 2.5 µl/hr; Alza Corporation), administering a constant supply of the different compounds, aseptically prefilled with either saline or DPIV inhibitor were placed subcutaneously in the abdominal area. Minipums were attached to a cannula via polyethylene tubing. The cannula was implanted intragastrically with a heating-induced enlarged tip of the cannula in the lumen of the gaster.

Quantification of macrometastasis on lungs

For quantification of lung surface colonies at later time points (2 weeks after tumor cell inoculation), en-bloc dissected lungs and the heart were injected with 8ml Bouin's solution (72% saturated picric acid solution, 23% formaldehyde, and 5% glacial acetic acid) and fixed in the same solution until lung surface nodules were counted. Three areas per lungs were examined using a gauge (1 cm^2) and lung surface colony numbers were expressed as mean / cm^2 according to the method of Wexler (Wexler, 1966).

Statistical analysis

Data from *in vivo* body weight gain and number of lung surface tumor colonies were analyzed by one-way ANOVAs and Fisher's PLSD post hoc tests, if appropriate. An asterisk indicates significant post hoc effects vs. saline (SHAM) treated controls obtained by Fisher's PLSD. All data are presented as means ± S.E.M.

Results

Effect of chronic infusion of isoleucyl thiazolidine fumarate (0mg, 0.04mg, 0.4mg, 4mg/24h) on lung tumor colonization

The change of body weight after chronic infusion of different dosages of isoleucyl thiazolidine fumarate in F344 rats 2 weeks after injection of MADB106 tumor cells is illustrated in Fig. 4. One factor ANOVA revealed a significant effect ($F(3,22) = 3.5$; $p = 0.03$) on body weight, which became significant in the post-hoc analysis at the 0.4mg and 4mg dosages.

The number of lung surface tumor colonies after chronic infusion of different dosages of isoleucyl thiazolidine fumarate in F344 rats 2 weeks after injection of MADB106 tumor cells is illustrated in Fig. 5. One factor ANOVA revealed a significant effect ($F(3,22) = 3.8$; $p = 0.03$) on colony numbers, which became significant in the post-hoc analysis at the 4mg dosage.

Effect of chronic infusion of isoleucyl thiazolidine fumarate, isoleucyl cyanopyrrolidine TFA, and valyl pyrrolidine fumarate on lung tumor colonization

The number of lung surface tumor colonies after chronic infusion of isoleucyl thiazolidine fumarate; isoleucyl cyanopyrrolidine TFA, and valyl pyrrolidine fumarate in F344 rats 2 weeks after injection of MADB106 tumor cells is illustrated in Fig. 6. One factor ANOVA revealed a significant effect ($F(3,20) = 3.8$; $p = 0.03$) on colony numbers, which became significant in the post-hoc analysis for isoleucyl cyanopyrrolidine TFA and isoleucyl thiazolidine fumarate compounds.

Discussion

Metastasis of MADB106 is reduced by chronic treatment using different DPIV inhibitors (isoleucyl thiazolidine fumarate; isoleucyl cyanopyrrolidine TFA) suggesting protective-like class effects. Possibly, isoleucyl thiazolidine fumarate and isoleucyl cyanopyrrolidine TFA protect from metastasis either via interaction with cell adhesion processes or via a modification of the cellular host defense mechanisms. It is also possible that DPIV inhibitor treatment exhibits cytostatic effects. These antimetastic

effects substantiate the biological properties of DPIV Inhibitors for the treatment of cancer and metastatic disease.

References

Cheng HC, Abdel-Ghany M, Zhang S, Pauli BU (1999) Is the Fischer 344/CRJ rat a protein-knock-out model for dipeptidyl peptidase IV-mediated lung metastasis of breast cancer?. Clin Exp Metastasis 17: 609-615

Mentlein R (1999) Dipeptidyl-peptidase IV (CD26)—role in the inactivation of regulatory peptides. Regul Pept 85: 9-24

Thompson NL, Hixson DC, Callanan H, Panzica M, Flanagan D, Faris RA, Hong WJ, Hartel-Schenk S, Doyle D (1991) A Fischer rat substrain deficient in dipeptidyl peptidase IV activity makes normal steady-state RNA levels and an altered protein. Use as a liver-cell transplantation model. Biochem J 273: 497-502

Tsuji E, Misumi Y, Fujiwara T, Takami N, Ogata S, Ikehara Y (1992) An active-site mutation (Gly^{633}—>Arg) of dipeptidyl peptidase IV causes its retention and rapid degradation in the endoplasmic reticulum. Biochemistry 31: 11921-11927

von Hörsten S, Helfritz A, Kuhlmann S, Nave H, Tschernig T, Pabst R, Ben-Eliyahu S, Meyer D, Schmidt RE, Schmitz C (2000) Stereological quantification of carboxyfluorescein-labeled rat lung metastasis: a new method for the assessment of natural killer cell activity and $_{tumor}$ adhesion in vivo and in situ. J Immunol Meth 239: 25-34

We claim:

1. Use of at least one inhibitor of dipeptidyl peptidase IV (DPIV) or DPIV-like enzyme activity for the preparation of a pharmaceutical composition for the treatment of cancer or a related disorder, wherein said inhibitor is selected from the group consisting of dipeptide compounds, peptide compounds comprising tri-, tetra- and pentapeptides, peptidylketones, aminoketone derivatives and side chain modified DP IV inhibitors.

2. The use according to claim 1, wherein the related disorder is metastasis.

3. The use according to claim 1, wherein the related disorder is tumor colonization.

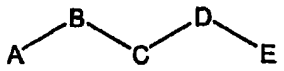
4. The use according to any one of the preceding claims, wherein the dideptidyl peptidase IV-like enzyme is selected from the group consisting of fibroblast activation protein α, dipeptidyl peptidase IV β, dipeptidyl aminopeptidase-like protein, N-acetylated α-linked acidic dipeptidase, quiescent cell proline dipeptidase, dipeptidyl peptidase II, attractin and dipeptidyl peptidase IV related protein (DPP 8), dipeptidyl peptidase 9 (DPP9), DPRP1, DPRP2, DPRP3 or KIAA1492.

5. The use according to any one of the preceding claims, wherein the structure of the dideptidyl peptidase IV-like enzyme is undiscovered.

6. The use according to any one of claims 1 to 4, wherein the inhibitor is a dipeptide compound formed from an amino acid and a thiazolidine or pyrrolidine group, and salts thereof.

7. The use according to claim 6 wherein the dipeptide compound is selected from the group consisting of L-*threo*-isoleucyl pyrrolidine, L-*allo*-isoleucyl thiazolidine, 1-*allo*-isoleucyl pyrrolydine, L-glutaminyl thiazolidine, L-glutaminyl pyrrolidine, L-glutamic acid thiazolidine, L-glutamic acid pyrrolidine and salts thereof.

8. The use according to any one of claims 1 to 4, wherein the inhibitor is a peptide compound useful for competetive modulation of dipeptidyl peptidase IV catalysis represented by the general formula



wherein

A is an amino acid except a D-amino acid;

B is an amino acid selected from Pro, Ala, Ser, Gly, Hyp, acetidine-(2)-carboxylic acid and pipecolic acid,

C is any amino acid except Pro, Hyp, acetidine-(2)-carboxylic acid, pipecolic acid and except N-alkylated amino acids, e.g. N-methyl valine and sarcosine,

D is any amino acid or missing, and

E is any amino acid or missing,

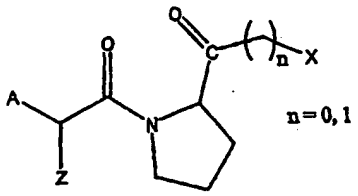
or:

C is any amino acid except Pro, Hyp, acetidine-(2)-carboxylic acid, pipecolic acid, except N-alkylated amino acids, e.g. N-methyl valine and sarcosine and except a D-amino-acid;

D is any amino acid selected from Pro, Ala, Ser, Gly, Hyp, acetidine-(2)-carboxylic acid and pipecolic acid, and

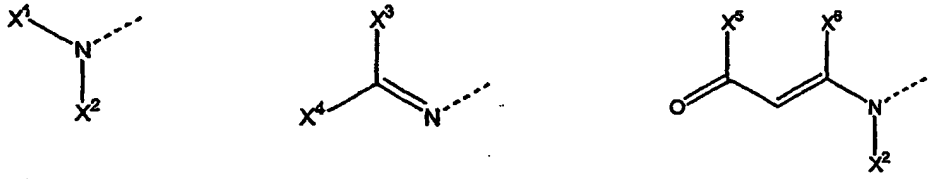
E is any amino acid except Pro, Hyp, acetidine-(2)-carboxylic acid, pipecolic acid and except N-alkylated amino acids, e.g. N-methyl valine and sarcosine.

9. The use according to any one of claims 1 to 4, wherein the inhibitor is a peptidylketone represented by the general formula



including all stereoisomers and pharmaceutically by acceptable salts thereof,

wherein
A is selected from :



and

X^1 is H or an acyl or oxycarbonyl group or an amino acid or peptide residue,

X^2 is H, $-(CH)_n$-NH-C_5H_3N-Y with n=2-4 or C_5H_3N-Y (a divalent pyridyl residue) and Y is selected from H, Br, Cl, I, NO_2 or CN,

X^3 is H or a phenyl or pyridyl residue, unsubstituted or substituted with one, two or more alkyl, alkoxy, halogen, nitro, cyano or carboxy residues,

X^4 is H or a phenyl or pyridyl residue, unsubstituted or substituted with one, two or more alkyl, alkoxy, halogen, nitro, cyano or carboxy residues,

X^5 is H or an alkyl, alkoxy or phenyl residue,

X^6 is H or an alkyl residue.

for n = 1

X is selected from: H, OR^2, SR^2, NR^2R^3, $N^+R^2R^3R^4$, wherein:

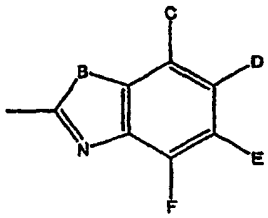
R^2 stands for acyl residues, which are unsubstituted or substituted with one, two or more alkyl, cycloalkyl, aryl or heteroaryl residues, or for all amino acids and peptidic residues, or alkyl residues, which are unsubstituted or substituted with one, two or more alkyl, cycloalkyl, aryl and heteroaryl residues,

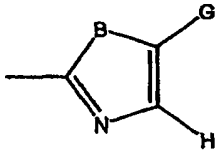
R^3 stands for alkyl and acyl functions, wherein R^2 and R^3 may be part of one or more ring structures of saturated and unsaturated carbocyclic or heterocyclic structures,

R^4 stands for alkyl residues, wherein R^2 and R^4 or R^3 and R^4 may be part of one or more ring structures of saturated and unsaturated carbocyclic or heterocyclic structures,

for n = 0

X is selected from:





wherein

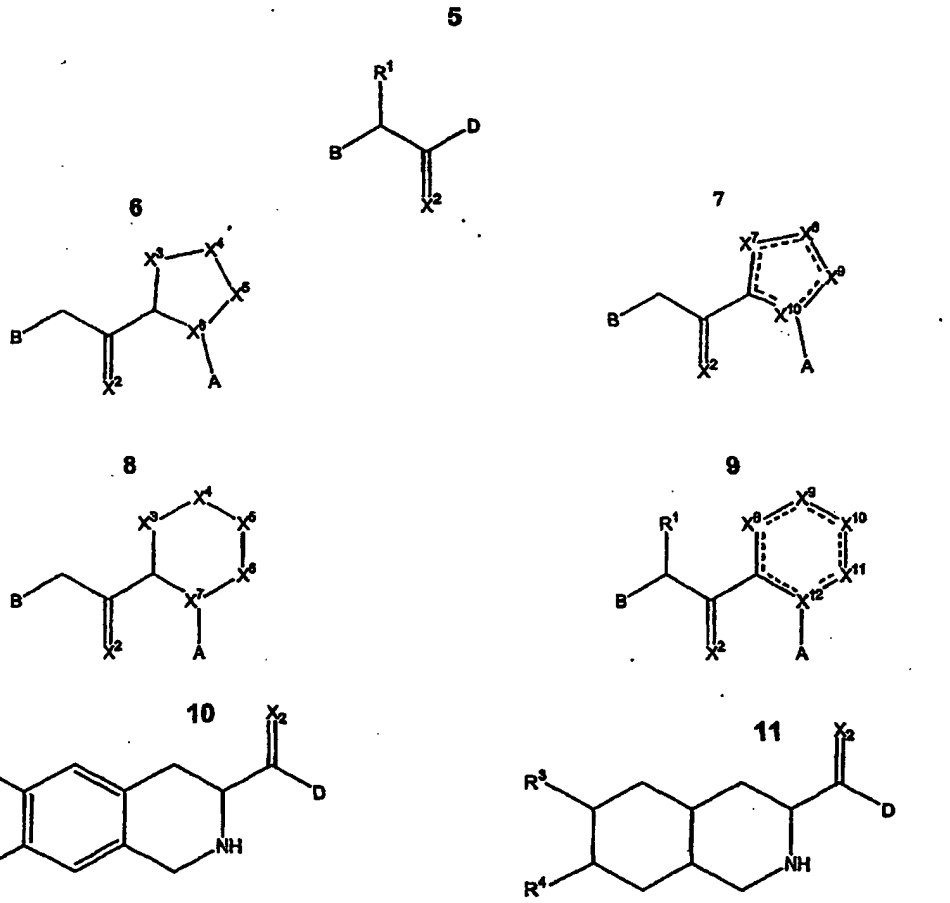
B stands for: O, S, NR^5, wherein R^5 is H, an alkyliden or acyl,

C, D, E, F, G, H are independently selected from unsubstituted and substituted alkyl, oxyalkyl, thioalkyl, aminoalkyl, carbonylalkyl, acyl, carbamoyl, aryl and heteroaryl residues; and

for n= 0 and n=1

Z is selected from H, or a branched or single chain alkyl residue from C_1-C_9 or a branched or single chain alkenyl residue from C_2-C_9, a cycloalkyl residue from C_3-C_8, a cycloalkenyl residue from C_5-C_7, an aryl- or heteroaryl residue, or a side chain selected from all side chains of all natural amino acids or derivatives thereof.

10. The use according to any one of claims 1 to 4, wherein the inhibitor is an aminoketone derivative represented by the general formulas 5, 6, 7, 8, 9, 10 and 11, including all stereoisomers and pharmaceutical acceptable salts thereof,



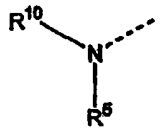
wherein:

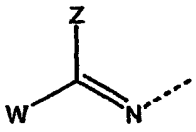
R^1 is H, a branched or linear C_1-C_9 alkyl residue, a branched or linear C_2-C_9 alkenyl residue, a C_3-C_8 cycloalkyl- , C_5-C_7 cycloalkenyl-, aryl- or heteroaryl residue or a side chain of a natural amino acid or a derivative thereof;

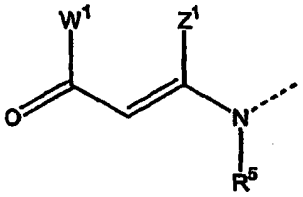
R^3 and R^4 are independently selected from H, hydroxy, alkyl, alkoxy, aryloxy, nitro, cyano or halogen,

A is H or an isoster of an carbonic acid, like a functional group selected from CN, SO_3H, CONHOH, $PO_3R^5R^6$, tetrazole, amide, ester, anhydride, thiazole and imidazole;

B is selected from:







wherein :

R^5 is H, $-(CH)_n$-NH-C_5H_3N-Y with n=2-4 and C_5H_3N-Y (a divalent pyridyl residue) with Y = H, Br, Cl, I, NO_2 or CN ,

R^{10} is H, an acyl, oxycarbonyl or a amino acid residue ,

W is H or a phenyl or pyridyl residue, unsubstituted or substituted with one, two or more alkyl, alkoxy, halogen, nitro, cyano or carboxy residues,

W^1 is H, an alkyl, alkoxy or phenyl residue,

Z is H or a phenyl or pyridyl residue, unsubstituted or substituted with one, two or more alkyl, alkoxy, halogen, nitro, cyano or carboxy residues,

Z^1 is H or an alkyl residue,

D is a cyclic C_4-C_7 alkyl, C_4-C_7 alkenyl residue which can be unsubstituted or substituted with one, two or more alkyl groups or a cyclic 4-7-membered heteroalkyl or a cyclic 4-7-membered heteroalkenyl residue,

X^2 is O, NR^6, $N^+(R^7)_2$, or S,

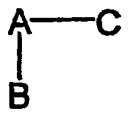
X^3 to X^{12} are independently selected from CH_2, CR^8R^9, NR^6, $N^+(R^7)_2$, O, S, SO and SO_2, including all saturated and unsaturated structures,

R^6, R^7, R^8, R^9 are independently selected from H, a branched or linear C_1-C_9 alkyl residue, a branched or linear C_2-C_9 alkenyl residue, a C_3-C_8 cycloalkyl residue, a C_5-C_7 cycloalkenyl residue, an aryl or heteroaryl residue,

with the following provisions:

Formula 6: X^6 is CH if A is not H,
Formula 7: X^{10} is C if A is not H,
Formula 8: X^7 is CH if A is not H,
Formula 9: X^{12} is C if A is not H.

11. The use according to any one of claims 1 to 4, wherein the inhibitor of DPIV or DPIV-like enzyme activity is represented by the general formula,



including all stereoisomers and pharmaceutical acceptable salts thereof,

wherein

A is an amino acid having at least one functional group in the side chain,

B is a chemical compound covalently bound to at least one functional group of the side chain of A, especially

- an oligopeptide having a chain length of up to 20 amino acids, or
- a polyethylene glycol having a molar mass of up to 20 000 g/mol,
- an optionally substituted organic amine, amide, alcohol, acid or aromatic compound having from 8 to 50 C atoms and

C is a thiazolidine, pyrrolidine, cyanopyrrolidine, hydroxyproline, dehydroproline or piperidine group amide-bound to A.

12. The use according to claim 11, wherein A is an amino acid, preferably an α-amino acid, especially a natural α-amino acid having at least one functional group in the side chain selected from the group consisting of threonine, tyrosine, serine, arginine, lysine, aspartic acid, glutamic acid or cysteine.

13. The use according to any one of the preceding claims, wherein said inhibitor is a pharmaceutical composition comprising a pharmaceutically acceptable carrier or diluent and a therapeutically effective amount of a said inhibitor or a pharmaceutically acceptable acid addition salt thereof.

14. The use according to any one of the preceding claims, wherein the inhibitor(s) is (are) used in combination with a pharmaceutically acceptable carrier and/or diluent.

Figure 1

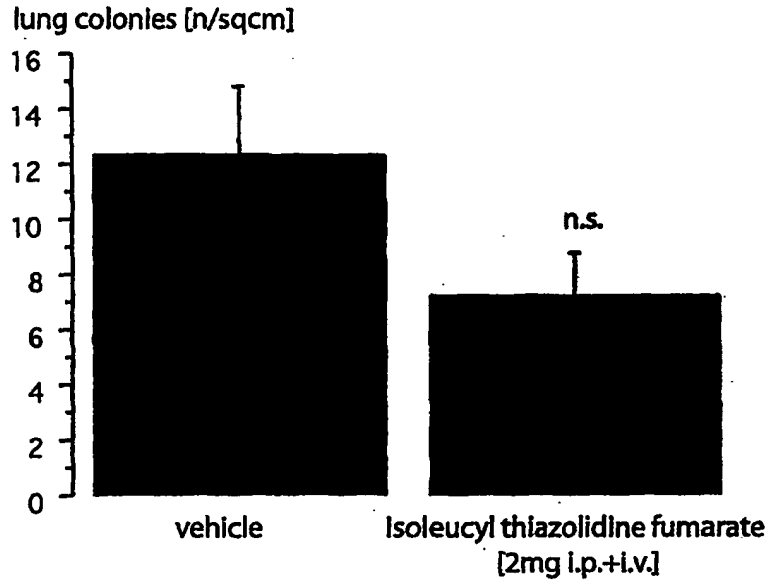

lung colonies [n/sqcm]
16
14
12
10
8
6
4
2
0
n.s.
vehicle
Isoleucyl thiazolidine fumarate
[2mg i.p.+i.v.]

Figure 2

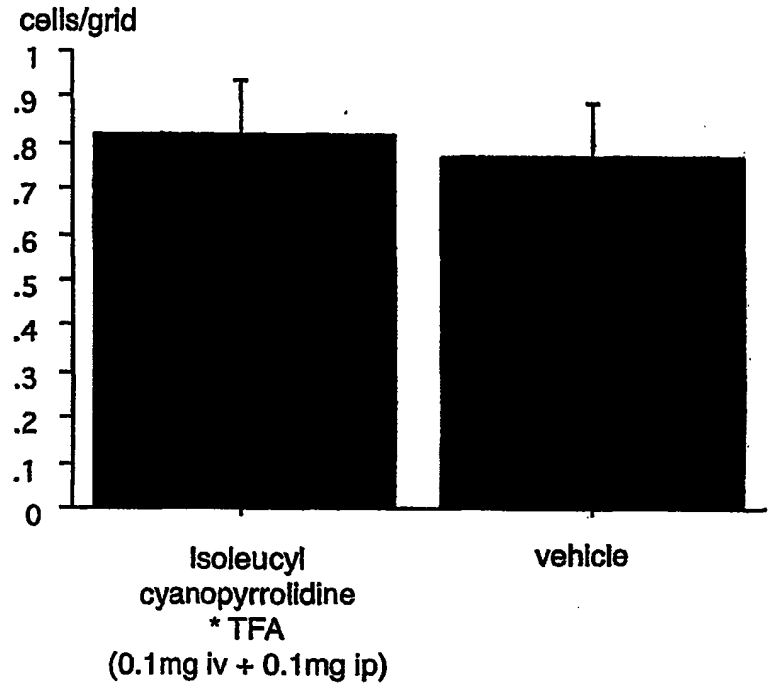

cells/grid
1
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
Isoleucyl cyanopyrrolidine * TFA (0.1mg iv + 0.1mg ip)
vehicle

Figure 3

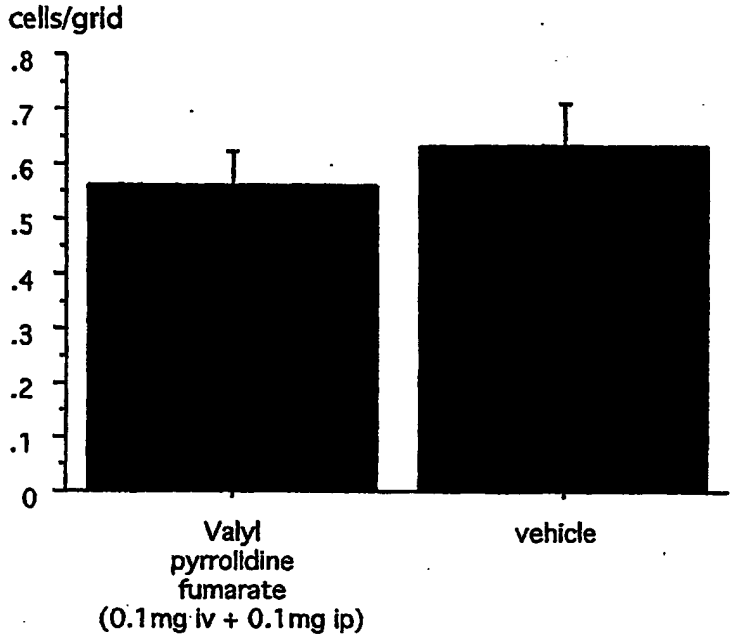

cells/grid
.8
.7
.6
.5
.4
.3
.2
.1
0
Valyl pyrrolidine fumarate (0.1mg iv + 0.1mg ip)
vehicle

Figure 4

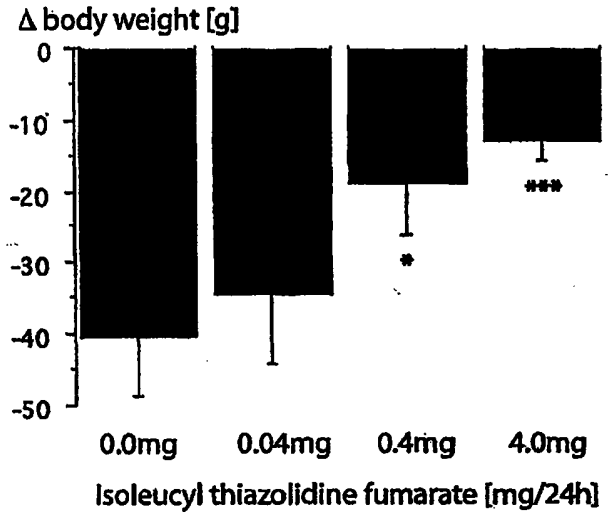

Δ body weight [g]
0
-10
-20
-30
-40
-50
*
***
0.0mg
0.04mg
0.4mg
4.0mg
Isoleucyl thiazolidine fumarate [mg/24h]

Figure 5

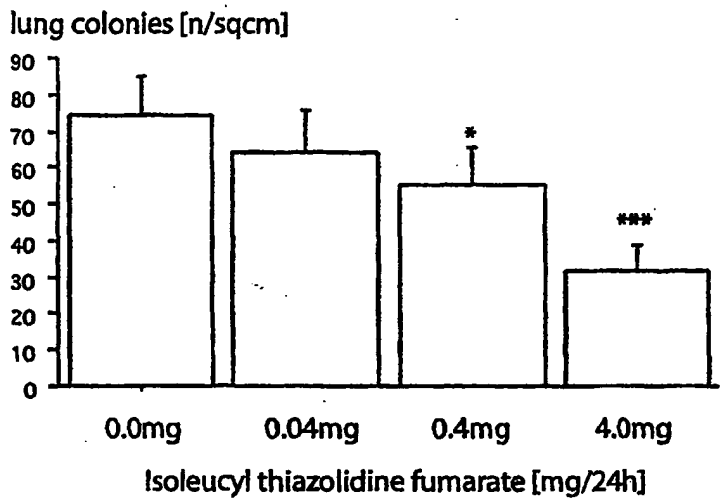
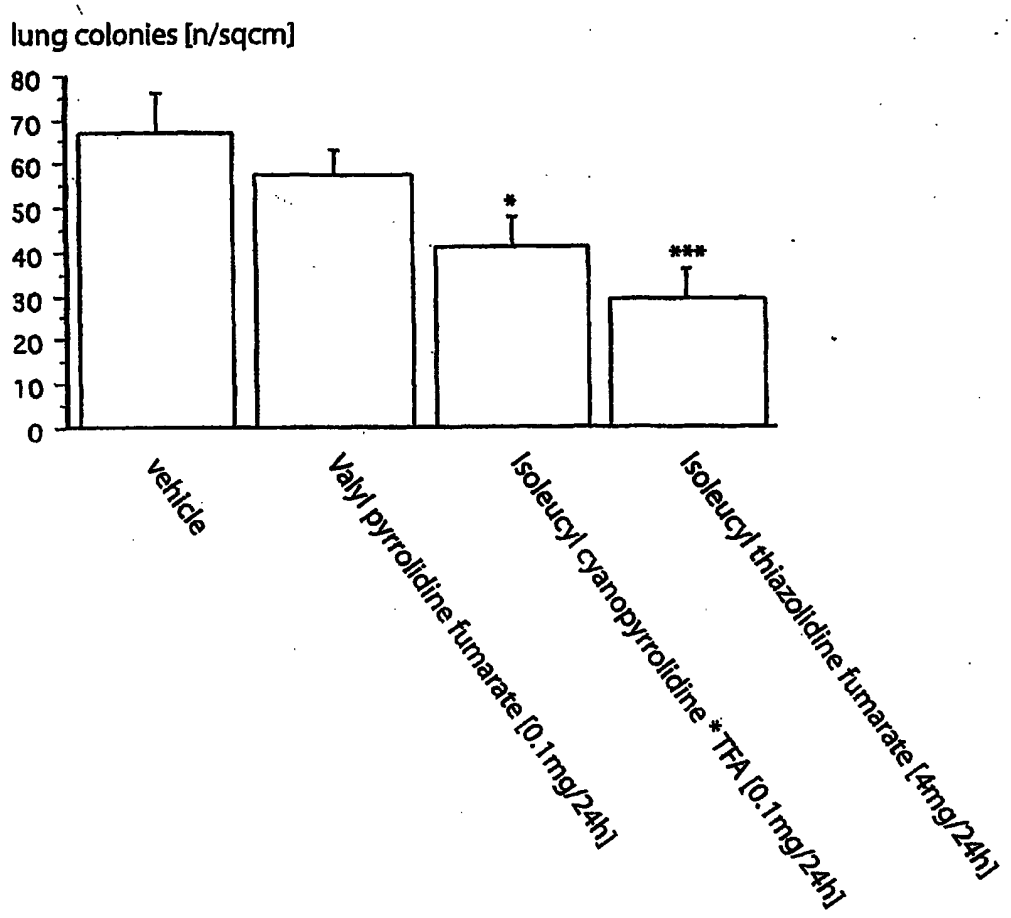

lung colonies [n/sqcm]
90
80
70
60
50
40
30
20
10
0
*
***
0.0mg
0.04mg
0.4mg
4.0mg
Isoleucyl thiazolidine fumarate [mg/24h]

Figure 6


lung colonies [n/sqcm]
80
70
60
50
40
30
20
10
0
*
***
vehicle
Valyl pyrrolidine fumarate [0.1mg/24h]
Isoleucyl cyanopyrrolidine *TFA [0.1mg/24h]
Isoleucyl thiazolidine fumarate [4mg/24h]